IDENTIFICATION OF NF-KAPPA B AND DNA-DEPENDENT PROTEIN KINASE (DNA-PK) AS NEW PLAYERS IN THE REGULATION AND SIGNALING OF THE ONCOGENIC PHOSPHATASE WIP1

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in Biochemistry and Molecular and Cellular Biology

By

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Identification of NF-kappaB (NF-κB) and DNA-dependent Protein Kinase (DNA-PK) as new players in the regulation and signaling of the oncogenic phosphatase Wip1

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Thesis Advisor: Albert J. Fornace, Jr., M.D.

ABSTRACT

Wild-type p53-induced phosphatase 1 (Wip1) is a stress-induced nuclear type 2C protein phosphatase (PP2C) that is over-expressed and amplified in many types of cancers. Further studies have shown Wip1 to act as an oncogene by inhibiting tumor suppressors such as p16/p19, p53, and ATM. The physiological role of Wip1 is to facilitate the return of the cell to homeostasis by inhibiting apoptosis and cell cycle arrest through the inactivation of several key stress signaling proteins such as p38, p53, and ATM. However, this physiological function may possibly be an additional oncogenic property of Wip1, since this may lead to premature inhibition of stress signaling and genomic instability in cases when Wip1 is over-expressed. Due to its oncogenic properties, Wip1 is an attractive drug target for human cancers, and, therefore, understanding Wip1 molecular functions is important. The studies outlined in this project identify novel regulation of Wip1 as well as novel Wip1 targets after stress and in a cancer setting. Nuclear factor-κappa B (NF-κB) directly induced or inhibited Wip1 expression at the transcriptional level depending on the cellular context. Additionally, Wip1 enhanced NF-κB activation in certain contexts by reducing the expression of the NF-κB inhibitor, Inhibitor of NF-κB-alpha (IκBα). DNA-PK, a major enzymatic
complex important for the Non-Homologous End-Joining DNA double strand break repair process, was identified as a Wip1 target. Specifically, Wip1 reduced phosphorylation levels of the catalytic subunit of DNA-PK (DNA-PKcs) at the activating residue Thr2609. As a consequence, Wip1 inhibited NHEJ after genotoxic stress. These results contribute to the understanding of the functions of Wip1 in both a physiological and tumorigenic setting. New roles for NF-κB and DNA-PK in Wip1 signaling were identified, which have direct implications in the understanding of Wip1 oncogenic functions and the treatment of human cancer.
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Note: Statistical significance (p-value < 0.05) is indicated by an asterisk (“*”) throughout
the dissertation and was determined by a T-test.
Chapter 1. Introduction.

1.1 Wip1 – Introduction

Wild-type p53-induced phosphatase 1 (Wip1) is a nuclear type 2C protein phosphatase (PP2C) that was discovered as a gene induced after ionizing radiation (IR) in a p53-dependent manner (5). Since the initial identification of Wip1, further investigation of its molecular functions indicate that the protein facilitates in returning the cell to homeostasis after stress (6). Reversing stress-induced pathways is essential for the survival of a cell after non-lethal stress exposure; however, proteins responsible for these functions, such as Wip1, are inevitably associated with uncontrolled cell growth and tumorigenesis in the event that their regulation goes awry. For instance, inhibition of cell cycle arrest and apoptosis caused by high levels of Wip1 expression may lead to genomic instability after stress, after which tumorigenesis may ensue. Wip1 has been found to be over-expressed and amplified in many types of cancers such as breast cancer, verifying an oncogenic role of Wip1(7-15) (Table 1). Furthermore, work performed using mouse models show that Wip1 accelerates tumor progression induced by a variety of oncogenic stresses such as myc, Hras1, and ErbB2 (1,2,16) (Figure 1). Therefore, unraveling the molecular functions of Wip1 is important since it is an attractive drug target.

The research regarding the mechanistic details of Wip1 involvement in stress signaling is somewhat incomplete. However, the existing literature describes several proteins that interact with Wip1. Furthermore, functional consequences of these protein interactions with Wip1 have been demonstrated and include the inhibition of cell cycle
<table>
<thead>
<tr>
<th>Organ type</th>
<th>DNA/RNA increase</th>
<th>p53 mutations</th>
<th>Prognosis</th>
<th>References</th>
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<tr>
<td>Breast adenocarcinoma</td>
<td>(11% CNG(^b), ECG(^c)) &lt;br&gt; (16% CNG; ECG) &lt;br&gt; (11% CNG; ECG) &lt;br&gt; (35% O(^d))</td>
<td>1/8 &lt;br&gt; 1/10</td>
<td>poorer</td>
<td>(9) &lt;br&gt; (12) &lt;br&gt; (13)</td>
</tr>
<tr>
<td>Ovarian clear cell carcinoma</td>
<td>(40% CNG; ECG)</td>
<td></td>
<td>poorer</td>
<td>(11)</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>(40% CNG; 28% O)</td>
<td>2/32</td>
<td>poorer</td>
<td>(14)</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>(51% CNG; 88% O) &lt;br&gt; (37% CNG; 27% O)</td>
<td></td>
<td>poorer</td>
<td>(17) &lt;br&gt; (18)</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>(74% O)</td>
<td></td>
<td></td>
<td>(10)</td>
</tr>
<tr>
<td>Pancreatic adenocarcinoma</td>
<td>(36% CNG)</td>
<td></td>
<td>poorer</td>
<td>(19)</td>
</tr>
</tbody>
</table>

\(^a\) Prognosis: “Poorer” indicates individuals with tumors increased Wip1 copy number and/or expression have significantly poorer prognosis than all individuals with that type of tumor.

\(^b\) CNG: Wip1 DNA copy number gain (compared to DNA in normal tissues)

\(^c\) ECG: increased Wip1 RNA expression significantly correlates with copy number gain

\(^d\) O: percentage of tumors with Wip1 RNA overexpression

**Table 1.**

Human tumors with Wip1 gene amplification and/or overexpression. **Adapted from (6).**

1.2 **Wip1 is a complementary oncogene.**

Wip1 gene amplification and over-expression has been associated with a number of different cancer types, the first of which to be identified is breast cancer (9,12)
Figure 1

Pivotal mouse model studies identifying Wip1 as a complementary oncogene. Deletion of PPM1D decreases A) Erbb2-induced and B) Hras1-induced mammary tumorigenesis (1), and myc-induced lymphomas (2). C) Deletion of PPM1D increases survival in APC\textsuperscript{\text{min}} mice, a model of colon cancer (3).

PPM1D is located within the chromosomal region 17q22-24, a genomic region that is commonly amplified (16-31%) in breast tumors (9,12,20), and amplified in a number of primary breast tumors and human breast cancer cell lines including the MCF7 cell line (2,9,12). Additionally, Wip1 over-expression is correlated with PPM1D gene amplification, since enhanced Wip1 expression was found in primary breast tumors positive for PPM1D amplification (13). Furthermore, the 17q21-24 region and targeted PPM1D amplification is associated with a poor patient prognosis and occurs in a number of other types of cancers such as ovarian clear cell adenocarcinomas (11,15),
neuroblastomas (14), medulloblastomas (17,18), gastric carcinoma (10), and pancreatic adenocarcinomas (19). The presence of Wip1 over-expression and *PPM1D* amplification in a substantial number of primary human tumors suggests that Wip1 plays a role in tumorigenesis.

There have been several pivotal molecular and genetic studies using mouse models that are consistent with the studies in primary human tumors and corroborate the role of Wip1 in cancer progression (summarized in Figure 1 and Table 2). Bulavin *et al* (9) showed that Wip1 null (*PPM1D/-*) mouse embryonic fibroblasts (MEFs) undergo premature senescence in culture and are less susceptible to oncogene-induced

<table>
<thead>
<tr>
<th>Evidence</th>
<th>References</th>
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<tr>
<td>1. Wip1 specifically inhibits p53 signaling by multiple mechanisms.</td>
<td>(1,9,21-24)</td>
</tr>
<tr>
<td>2. Wip1 inhibits the activity of other tumor suppressors (ARF, p16^INK4A^).</td>
<td>(1)</td>
</tr>
<tr>
<td>3. Wip1 abrogates DNA damage response pathways and cell cycle checkpoints.</td>
<td>(10,23,25)</td>
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<td>4. Wip1 can transform primary rodent fibroblasts in conjunction with other oncogenes.</td>
<td>(9,10,13)</td>
</tr>
<tr>
<td>5. Wip1 accelerates tumorigenesis in a mammary tumor susceptible model.</td>
<td>(26)</td>
</tr>
<tr>
<td>6. Wip1 is amplified and overexpressed in multiple types of human tumors.</td>
<td>(7,9,11,13,14,16-19,27)</td>
</tr>
<tr>
<td>7. Wip1 amplification and overexpression is often associated with poorer prognosis.</td>
<td>(11,16-19)</td>
</tr>
<tr>
<td>8. Wip1 null primary embryo fibroblasts are resistant to transformation by oncogenes.</td>
<td>(1)</td>
</tr>
<tr>
<td>9. Wip1 null mice are resistant to spontaneous and oncogene-induced tumors.</td>
<td>(1,10,28)</td>
</tr>
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</table>

**Table 2**

*Evidence that Wip1 is an oncogene.* Adapted from (6).

Wip1 in cancer progression (summarized in Figure 1 and Table 2). Bulavin *et al* (9) showed that Wip1 null (*PPM1D/-*) mouse embryonic fibroblasts (MEFs) undergo premature senescence in culture and are less susceptible to oncogene-induced
transformation. PPM1D-/mice have lower
tumorigenecity when injected into nude mice than the transformed wild type MEFs (9).
Additionally, PPM1D-/ mice have a decreased occurrence of mammary tumorigenesis
with over-expression of ErbB2 and Hras1 than control mice, and the mechanism is
through inhibition of the p38 and p16\textsuperscript{Ink4a}/p19\textsuperscript{Arf} pathway (Figure 1) (1). A separate study
demonstrates that PPM1D-/ mice also have a significantly lower occurrence of
spontaneous tumors (mostly lymphomas) compared to PPM1D+/+ mice and PPM1D+/-
mice (29). Moreover, PPM1D-/ mice are more resistant to myc-induced lymphomas
compared to control mice; the percentage of tumor-free mice that over-express myc in B-cells is significantly higher in mice deficient in Wip1 than control mice (Figure 1) (2).
More recently, Wip1 was shown to inhibit apoptosis in intestinal stem cells through p53,
which promotes polyp formation in a model of colorectal cancer (Figure 1) (3). Taken
together, these studies show that Wip1 facilitates tumorigenesis of various tissues by
cooperating with oncogenes.

The results described above provide overwhelming evidence that Wip1 has
oncogenic properties and facilitates tumorigenesis. However, mice that over-express
Wip1 in the mammary epithelia do not show an increase in tumorigenesis (16), indicating
that Wip1 over-expression alone is not oncogenic (at least in mouse mammary tissue). Hence, Wip1 has been termed a complementary oncogene, namely a gene that only
promotes tumorigenesis when over-expressed in parallel with other oncogenic stresses.
1.3 Wip1 transcriptional regulation.

Wip1 was discovered on the basis that it is a transcriptional target of p53 (5). Since then, there have been studies elaborating on upstream mechanisms of p53-dependent Wip1 transcriptional regulation. The distinct upstream mechanisms responsible for Wip1 expression seem to depend upon the type of genotoxic stress exposed to cells, such as ionizing radiation (IR) and ultraviolet radiation (UVR).

However, with the exception of a few studies, there has been limited research focused on additional mechanisms, including those independent of p53, involved in the regulation of Wip1 expression after stress. To date, the known mechanisms of Wip1 transcriptional regulation include those dependent on p53, CREB, E2F1, and Estrogen Receptor-alpha (ERα) (6,30).
1.3.1 Transcriptional regulation mediated by p53 and CREB.

It is clear that p53 is a transcriptional regulator of Wip1. Not only was Wip1 discovered as a p53 target after IR (5), but Takekawa et al showed an increase in Wip1 mRNA levels in response to DNA damaging agents including IR, UVR, and methyl methane sulfonate (MMS), which was dependent on p53 (24). The precise mechanisms upstream of p53 involved in Wip1 expression regulation differs depending on the type of stress. p38 modulates Wip1 expression upstream of p53 specifically after UVR exposure, since UVR-irradiated p53-proficient cells treated with a p38 inhibitor failed to display an increase in Wip1 mRNA levels (24). However, Wip1 transcription is not p38/p53-dependent after IR, since the increase in Wip1 transcript is unaffected in IR-exposed A549 cells that are treated with a p38 inhibitor (24). This is not surprising due to the disparity in stress signaling following various stresses, especially those responsible for activating p53 (31-33). For example, p38 is a predominate player in activating p53 and the G2/M checkpoint signaling after UVR (34), while the ataxia-telangiectasia mutated (ATM) kinase acts to coordinate both checkpoint and apoptosis signaling after IR but not UVR (35).

More recent studies on p53-regulated Wip1 expression reveal that p53 not only regulates *PPM1D* transcriptionally, but it also regulates Wip1 expression post-transcriptionally. Rossi et al identified p53 response elements (p53RE) in the *PPM1D* proximal promoter and 5’ untranslated region (5’UTR) (36). In response to UVR and IR, there is a p53-dependent shift in the transcriptional start site of *PPM1D* – p53 shifts to the p53RE in the 5’UTR, which creates shortened *PPM1D* mRNA. This indicates that p53
post-transcriptionally regulates Wip1 expression since shorter transcripts are more efficiently exported out of the nucleus (36).

In the same study as above, CREB was identified as a transcription factor that positively regulates basal *PPM1D* transcription in colon cancer HCT-116 cells (36). Additionally, CREB regulation of *PPM1D* transcription was shown to be independent of p53, since CREB was bound to the *PPM1D* promoter region in both wild type and p53-/- HCT-116 cells (36). CREB also modulates IR-induced Wip1 expression, since there was an increase in CREB association with the *PPM1D* promoter after IR and UVR in these cells. Furthermore, CREB appears to co-regulate IR- and UVR-regulated *PPM1D* transcription with p53, since the increased association of CREB with the *PPM1D* promoter was absent in HCT-116 p53-/- cells. Thus, CREB appears to positively modulate Wip1 expression basally and coordinates with p53 to upregulate Wip1 expression after genotoxic stress (36).

**1.3.2 Transcriptional regulation mediated by E2F1.**

To date, there has been little research focused on p53-independent regulation of Wip1 expression. However, there is evidence supporting the existence of a p53-independent mode of Wip1 regulation. For example, treatment with anisomycin, a protein synthesis inhibitor and stress signaling activator (37), induced Wip1 transcription in both A549 (p53-proficient) and H1299 (p53-deficient) cells (24). Indeed, Hershko *et al* showed that, independent of p53, *PPM1D* is a target of the transcription factor E2F1, which is involved in stress-induced apoptosis and cell cycle checkpoint signaling (38).
This was discovered using cells overexpressing E2F1 and that have depleted p53. However, it is worth noting the conclusion that stress-induced E2F1 activation leads to p53-independent Wip1 transcriptional regulation was made based upon the fact that E2F1 is known to be activated in response to stress, even though the cells in these experiments only over-expressed E2F1 and were not exposed to stress (38). Thus, while E2F1-dependent and p53-independent transcriptional regulation of Wip1 is conclusive, it remains unclear whether this mechanism is activated after exogenous stress.

1.3.3 Transcriptional regulation mediated by Estrogen Receptor alpha (ERα).

Several pieces of evidence from the literature indicate that Wip1 may be regulated in a steroid-dependent manner, and there is a single report identifying PPM1D as a target gene of ERα (30). First, Wip1 is over-expressed in a number of cancers regulated by steroids such as breast cancer and ovarian cancer (6). Additionally, a large portion of primary breast tumors that have Wip1 over-expressed also have a high expression of ERα (8). Han et al showed that Wip1 is regulated by ERα, since Wip1 expression increased with 17-β-Estradiol (E2) incubation and ectopic expression of ERα (30). Additionally, ERα binds to the promoter region of PPM1D, and regulation of Wip1 by ERα was shown to increase the transcription of ERα genes (including its own PPM1D gene) by enhancing the interaction of ERα with the coactivator, steroid receptor coactivator-1 (src-1). This study indicates that in steroid-regulated cancers, Wip1 is upregulated by ERα and then elicits pro-tumorigenic effects by enhancing E2 signaling through ERα and promoting cell cycle progression, at least in breast cancer cells (30).
<table>
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<th>Protein</th>
<th>Context of Wip1 inhibition</th>
<th>Dephosphorylation Residues</th>
<th>Functional consequence</th>
<th>Reference</th>
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<tr>
<td>Chk2</td>
<td>Inactivates Chk2</td>
<td>S19, S33/S35, T68, T432</td>
<td>reduces IR-induced Chk2-dependent apoptosis; dulls G2/M checkpoint</td>
<td>(22,44,45)</td>
</tr>
<tr>
<td>p38</td>
<td>Inactivates p38 after UVR</td>
<td>T180</td>
<td>reduces apoptosis; dulls cell cycle checkpoints after UVR</td>
<td>(24)</td>
</tr>
<tr>
<td>ATM</td>
<td>Reduces ATM activity after IR</td>
<td>S1981 (human) S1987 (mouse)</td>
<td>reduces apoptosis and dulls cell cycle checkpoints after IR; enhances Eμ-myc-induced lymphoma</td>
<td>(2)</td>
</tr>
<tr>
<td>chk1</td>
<td>Reduces chk1 activity after UVR</td>
<td>S345, and to a lesser extent, S317</td>
<td>reduces apoptosis and dulls cell cycle checkpoints after IR</td>
<td>(23)</td>
</tr>
<tr>
<td>p53</td>
<td>Reduces activity after IR and UVR</td>
<td>S15</td>
<td>reduces UVR and IR-induced apoptosis</td>
<td>(23)</td>
</tr>
<tr>
<td>UNG2</td>
<td>Reduces activity after UVR</td>
<td>T6</td>
<td>suppresses BER</td>
<td>(25)</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Reduces activity after cytokine stimulation</td>
<td>S536</td>
<td>suppresses inflammatory response</td>
<td>(46)</td>
</tr>
<tr>
<td>MDM2/X</td>
<td>Stabilizes protein after IR</td>
<td>S395/S403</td>
<td>Suppression of p53 effects</td>
<td>(21,47)</td>
</tr>
</tbody>
</table>

Table 3

Summary of the mechanism and consequence of Wip1 inhibition of stress signaling proteins.
1.4 Wip1 inhibition of stress signaling proteins.

Cells have adapted a way for normal, “everyday” functions, such as those involved in cell cycle progression, to temporarily shut down while stress-induced damage is repaired. Hence, depending on the cell cycle phase of the cell during the time of damage, the cell can halt cell cycle progression by eliciting checkpoints (39,40). However, if the damage is not repairable, then the cell will trigger apoptosis. Apoptosis and cell cycle arrest are crucial, since cells that have a dysfunctional checkpoint and apoptotic response are able to replicate despite persistent damage, which can potentially lead to genomic mutations and tumor formation. Therefore, there must be signaling proteins responsible for both the initiation of cell cycle arrest during damage repair (or apoptosis if damage is not repaired) and turning off these pathways upon the completion of repair to allow the cell back into normal cell cycle progression – Wip1 appears to play a role in the latter process by inactivating a number of key proteins, which will be discussed below (see Table 3 and Figure 3).

1.4.1 p38 MAPK

The first established target of Wip1 was p38. p38 is a member of the MAPK family and is one of the main sensor proteins activated by phosphorylation at two residues, T180 and Y182, in response to various stresses including UVR, and functions in cell cycle checkpoint signaling (41-43). After exposure of a cell to UVR, for example, p38 is activated and initiates signaling resulting in the phosphorylation of CDC25B at the site of 14-3-3 binding, and CDC25B is then sequestered in the cytoplasm where it is
unable to activate Cdk1 thereby prohibiting the cell to progress through G2 and into mitosis (39,41,42,48). Evidence suggests that Wip1 seems to be involved in reversing the p38-dependent checkpoint response after UVR by inactivating p38 by direct dephosphorylation (5,24). p38 dephosphorylation by Wip1 specifically on T180 (pT180-p38) was validated by Yamaguchi et al using in vitro phosphatase assays with the catalytic subunit of Wip1 (amino acids 1-420) and a p38 diphosphopeptide (pT180, pY182; the kinetic parameters of this reaction were measured as $K_m$ 13$\mu$M, $K_{cat}$ 1.7 s$^{-1}$, and $K_{cat}/K_m$ 131 (10$^3$ x M$^{-1}$ s$^{-1}$; Table 3 and Table 4) (49). The functional effects of

<table>
<thead>
<tr>
<th>Wip1 Substrate</th>
<th>Sequence</th>
<th>Kinetic Parameters</th>
<th>Found in vivo?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$K_m$ (µM)</td>
<td>$K_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>p38α (180pT, 182pY)$^a$</td>
<td>TDDEMpTGpYVAT</td>
<td>36±3</td>
<td>1.4±0.04</td>
</tr>
<tr>
<td>ATM (1981pS)$^a$</td>
<td>AFEEGpSQSTTI</td>
<td>26±2</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td>p53 (15pS)$^a$</td>
<td>VEPPLpSQETF5</td>
<td>25±2</td>
<td>1.3±0.03</td>
</tr>
<tr>
<td>Chk1 (345pS)$^a$</td>
<td>QGISFpSQPTCPD</td>
<td>187±63</td>
<td>1.8±0.5</td>
</tr>
<tr>
<td>H2AX (139pS)$^a$</td>
<td>KATQApSQEY</td>
<td>40±3</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>UNG2(6pT 8pY)$^b$</td>
<td>KpTLpYS-NH2</td>
<td>20±2</td>
<td>0.8±0.03</td>
</tr>
</tbody>
</table>

Table 4

**Kinetic parameters for in vitro wip1 dephosphorylation of various substrates.** Bold letters indicate phosphorylated sites. $^a$ Adapted from (2). $^b$ Adapted from (49).
Wip1 inactivation of p38 are not only the suppression of its own transcription in a negative feedback loop with the p38-p53 pathway, but also the inhibition of other actions of p38 such as cell cycle checkpoint signaling (Figure 3).

1.4.2 Chk1/2

The Checkpoint kinases 1 and 2 (Chk1 and Chk2) are transducer proteins activated after exposure of cells to various DNA-damaging agents including UVR and IR, and are involved in coordinating cell cycle checkpoint and apoptosis initiation (35). Both proteins are activated by phosphorylation on specific residues – T68 on Chk2 and S345 on Chk1 – by the DNA damage sensor proteins ATM and ataxia-telangiectasia and Rad3-related (ATR), respectively. After activation, Chk1 and Chk2 phosphorylate members of the CDC25 family, which results in the inactivation of Cdk5 and subsequent cell cycle arrest (Figure 3) (35,39). In addition to the initiation of checkpoints, Chk1 and Chk2 play roles in checkpoint maintenance, mainly by phosphorylating and activating p53, which results in the transcription of p21 and inhibition of the transcription of genes important in cell cycle progression (35).

Like p38, Wip1 can inactivate and dephosphorylate both Chk1 and Chk2 after IR and UVR. Incubation of immunopurified Chk1 from UVR-treated U2OS cells and pure Wip1 protein resulted in a decrease in Chk1 activity (23). Additionally, in vitro and in vivo studies by Lu et al show that Wip1 interacts with and directly dephosphorylates Chk1 on pS345 (23) (Table 3 and Table 4). However, dephosphorylation and inactivation of Chk1 by Wip1 does not occur only after UVR, since E1A/Ras/PPM1D−/−
MEFs have distinctly lower pS345-Chk1 levels compared to wild type E1A/Ras MEFs after IR (23). Although Chk2, and not Chk1, is thought to be the predominate transducer protein activated in response to IR-induced DSBs, recent work demonstrates that Chk1 and Chk2 are differentially regulated depending upon the cell cycle phase after IR (50,51) and that there is cross-talk among these pathways (35,39). Therefore, Wip1 may possibly inactivate Chk1 after both IR and UVR (Figure 3).

Similar studies indicate that Wip1 also dephosphorylates and inactivates Chk2. Wip1 and Chk2 were found to interact in vitro and in vivo via studies performed with
tagged Chk2 and Wip1 proteins, and this interaction is dependent on the intrinsic kinase activity of Chk2 (44,45). Fujimoto et al and Olivia-Trastoy et al independently demonstrated that Wip1 inactivates Chk2 after IR probably through direct dephosphorylation of Chk2 on T68 (pT68-Chk2) (22,44) (Figure 3, Table 3, Table 4). However, previous work also illustrates that Wip1 dephosphorylates Chk2 at other residues (22); therefore, while Wip1-dependent Chk2 inactivation coincided with dephosphorylation of Chk2 on T68, inactivation by Wip1 dephosphorylation of any of these other residues on Chk2 cannot be excluded. It also should be noted that the studies showing Chk1/2 as Wip1 targets were performed in vitro and need to be confirmed in vivo.

1.4.3 ATM

ATM is a main sensor protein activated in response to DSBs such as those resulting from IR. After DSB detection, ATM undergoes autophosphorylation on several sites including S1981, which is a site important for converting the protein into its active form (35). Once activated, ATM aids in coordinating cell cycle arrest, along with DNA repair and, if necessary, apoptosis. Particularly, ATM phosphorylates and activates Chk2 and p53 to facilitate checkpoint and apoptosis initiation (Figure 3) (40).

Since S1981 phosphorylation is an important event in ATM activation, dephosphorylation of this residue results in ATM inactivation and attenuation of ATM-dependent cell cycle arrest (35). There is evidence for a role of Wip1 in ATM dephosphorylation and inactivation. Levels of pS1981-ATM after IR increases in

15
E1A/Ras PPM1D<sup>−/−</sup> MEFs compared to control cells, and overexpression of Wip1 in E1A/Ras MEFs results in decreased amounts of pS1981- ATM after IR (2). Similar results from experiments using human cancer cell lines also demonstrate that Wip1 directly dephosphorylates pS1981 on ATM, and <i>in vitro</i> phosphatase reactions using a phosphopeptide of ATM (pS1981) and pure Wip1 protein have a calculated $K_M$ of 26μM, $K_{cat}$ of 1.6 s<sup>−1</sup> and a $K_{cat}/K_m$ of 64 (10<sup>3</sup> x M<sup>−1</sup> s<sup>−1</sup>) (Table 3 and Table 4) (2). Furthermore, Wip1 accelerated tumor formation of Eµ-myc-driven lymphoma in mice in an ATM- and p53-dependent manner (2). Therefore, dephosphorylation and inhibition of ATM provides an additional mechanism by which Wip1 reverses signaling after genotoxic stress and promotes tumorigenesis (Figure 3).

1.4.4 p53

p53 is a transducer protein and tumor suppressor that facilitates both apoptosis and cell cycle checkpoint maintenance. After IR, ATM and ATR signaling leads to activation of p53 by phosphorylation on many residues including S15 either directly or indirectly through activation of Chk2 and Chk1 (35). p53 also participates in UVR-induced checkpoint signaling after activation by ATR and p38 (52). In the active form, p53 acts as a transcription factor to upregulate the transcription of proteins, such as p21, involved in apoptosis and the maintenance of cell cycle checkpoints (Figure 3) (35).

Wip1 indirectly leads to p53 inactivation after UVR and IR by inactivating p38, ATM, Chk1, and Chk2 (as mentioned above); however, there is also evidence that Wip1 directly inactivates p53 through dephosphorylation of pS15. Pure Wip1 dephosphorylates pS15-p53 immunopurified from UVR-treated U2OS cells as well as a
p53 phosphopeptide (pS15) *in vitro* with a $K_m$ of 25$\mu$m, a $K_{cat}$ of $1.3 \text{s}^{-1}$, and a $K_{cat}/K_m$ of 51 ($10^3 \times M^{-1} \text{s}^{-1}$) (Table 3 and Table 4) (2). Furthermore, cells with reduced or increased Wip1 expression have higher or lower levels, respectively, of pS15-p53 after IR and UVR than control cells (23). These data indicate that Wip1 dephosphorylates pS15-p53 after genotoxic stress, which, since phosphorylation on S15 is necessary for full protein stabilization and activation, suggests that Wip1 inactivates p53.

Phosphorylation on other sites of p53 besides S15 is also important for maximum p53 stabilization, and work indicates that phosphorylation at some of these other sites are also affected by Wip1 – S33 is an example. Phosphorylation of S33 is inhibited after UVR by Wip1 over-expression in H1299 cells (transfected with p53); however, *in vitro* phosphatase assays reveal that Wip1 does not dephosphorylate S33 on p53 (24). Treatment of cells with the p38 inhibitor SB203580, on the other hand, effectively reduced the levels of pS33-p53 after UVR, indicating that Wip1 probably decreases S33 phosphorylation through exerting negative feedback on the p38 pathway (24,34). Thus, seemingly through a variety of both direct and indirect mechanisms, Wip1 reduces p53 phosphorylation and subsequent activation after stress.

### 1.4.5 $p16^{\text{ Ink4a }}$ and $p19^{\text{ Arf }}$

$p16^{\text{ Ink4a }}$ and $p19^{\text{ Arf }}$ are proteins activated by sustained mitogenic signaling that function to facilitate cell cycle arrest (53,54). Briefly, $p16^{\text{ Ink4a }}$ signaling ultimately inhibits S phase progression by inhibiting Cdk activity, whereas $p19^{\text{ Arf }}$ facilitates p53 activation by negatively regulating Mdm2 and also leads to cell cycle arrest (Figure 3).
Due to these tumor suppressor properties, $p16^{Ink4a}$ and $p19^{Arf}$ are commonly down-regulated in cancers (53,54), and Wip1 seems to augment this effect.

Wip1 increases tumorigenesis by acting through the $p16^{Ink4a}$-$p19^{Arf}$ pathway. $PPM1D^{-/-}$ mice are resistant to H-ras1 and ErbB2-induced mammary tumorigenesis. Sensitivity to H-ras1 and ErbB2 oncogenic stresses is restored in $PPM1D^{-/-}$ mice treated with a p38 inhibitor and in $PPM1D^{-/-}/p16^{-/-}$ and $PPM1D^{-/-}/p19^{-/-}$ genetic backgrounds, suggesting that Wip1 inhibits $p16^{Ink4a}$- and $p19^{Arf}$-mediated cell cycle arrest through inactivation of p38 (Figure 3). Additionally, expression levels of $p16^{Ink4a}$ and $p19^{Arf}$ are lower in ErbB2-driven tumors in $PPM1D^{-/-}$ mice compared to those generated in control mice (1). Furthermore, $p16^{Ink4a}$ has been shown to exist in low levels in human breast cancer tumors that over-express Wip1 (8). Taken together, these data indicate that Wip1 functions as an oncogene in part through p38 inactivation and subsequent suppression of the $p16^{Ink4a}$-$p19^{Arf}$ pathway.

1.4.6 MDM2/X

MDM2 and MDMX, like Wip1, are transcriptional targets of p53. Upregulation of MDM2/X leads to an autoregulatory negative feedback loop, since MDM2/X is an E3 ubiquitin ligase that binds to p53 and leads to the ubiquitination and degradation of the p53 protein (55,56). Because of its negative regulation of p53, MDM2/X facilitates turning the DNA damage response off after stress and is thought to positively regulate tumorigenesis. It is fitting with Wip1 function, therefore, for Wip1 to enhance MDM2/X and its negative effects on p53 signaling. Lu et al show that Wip1 directly
dephosphorylated MDM2/X on serine 395, which is a site that is phosphorylated by ATM after genotoxic stress (Table 3) (21). As a result, Wip1 stabilizes the MDM2/X protein, which enhances p53 degradation. Additionally, Zhang et al confirmed the direct dephosphorylation of S395 of MDM2 by Wip1, and also showed that Wip1 directly and indirectly dephosphorylates MDMX on multiple residues (47). Wip1 directly dephosphorylated S403 of MDMX and indirectly lead to the dephosphorylation of S342 and S376 of MDMX (Table 3) (47). However, the studies showing MDM2 and MDMX as Wip1 targets were done in vitro and need to be confirmed in vivo. Therefore, Wip1 dephosphorylation of MDM2/X and enhancement of MDM2/X-dependent p53 degradation provides an additional mechanism by which Wip1 returns the cell to homeostasis after stress and promotes tumorigenesis (Figure 3).

1.4.7 Wip1 inhibits the inflammatory response through NF-κB

Analysis of the phenotype of Wip1-/- mice provided the first clue that Wip1 may play a role in inflammation. Wip1-/- mice are more susceptible to infection and have a higher frequency of ulcerated skin lesions, and splenic lymphocytes harvested from Wip1-/- mice show a dampened proliferative response after stimulation with phytohemagglutinin (PHA) and lipopolysaccharide (LPS) compared to the wild type controls (27). Indeed, inhibition of inflammation was reported as an additional function of Wip1 by Chew et al (46). Wip1 was shown to suppress the expression of targets of the transcription factor Nuclear Factor-kappa B (NF-κB) after cytokine stimulation. Two mechanisms were identified – one mechanism is through direct inhibition of NF-κB and the other is through inhibition of a previously identified Wip1 target, p38. Wip1 inhibits
NF-κB activity not by altering the translocation into the nucleus or the binding to DNA of the NF-κB subunits but by directly dephosphorylating S536 of the p65 subunit (Table 3). Inhibition of cytokine-induced p38 activity by Wip1 is presumably through its previously defined function – dephosphorylation of T180 of p38. The functional consequence of Wip1 inhibition of cytokine-induced NF-κB and p38 activity was shown to be a reduction in the expression of NF-κB target genes such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNFα), and IL-8. Additionally, splenocytes from LPS-injected Wip1−/− mice show a “hyperactivated” phenotype (46). Therefore, Wip1 appears to play a role in inflammation similar to that after genotoxic stress, i.e. Wip1 helps to turn-off the inflammatory response (Figure 3).

1.4.8 Wip1 relieves stress-induced cycle checkpoints.

There have been various functional studies validating a role for Wip1 in the G1/S, intra-S, and G2/M checkpoint. Results from several groups show that Wip1 inhibits G2/M cell cycle arrest through inhibition of Chk2 after both IR and UVR (23,44). Additionally, flow cytometry analysis of PPM1D−/− versus wild type MEFs reveal that MEFs deficient in Wip1 have a significantly higher G2:M ratio (52.7) compared to the wild type (17.4), indicating that Wip1 plays a role in the transition from the G2 cell cycle phase to mitosis (27).

In addition to the G2/M checkpoint, Wip1 has been shown to function in the G1 checkpoint. Synchronized PPM1D−/− MEFs exhibit an exaggerated G1 arrest after IR compared to wild type, which is due to a higher p53 activity level (27). These data
indicate that Wip1 probably participates in the reversal of signaling important for the maintenance, and not initiation, of the G1 checkpoint (through p53 inhibition) since there was no difference at earlier time points following IR. Hence, Wip1 helps to return the cell to normal cell cycle progression after stress by decreasing p53 activity, and thereby negatively regulating pathways involved in maintaining the G1/S cell cycle arrest.

Finally, evidence suggests that Wip1 has a function in the intra S-phase cell cycle checkpoint after stress. Lu et al showed that Wip1 plays a role in regulating the intra-S phase after IR and UVR by using cells either over-expressing Wip1 or that have Wip1 knocked down by siRNA (23). These results show that Wip1 assists in returning cells to normal cell cycle progression by dulling not only the G2/M and the G1/S checkpoints, but also the intra-S phase checkpoint.

1.5 Wip1 inhibits apoptosis.

Apoptosis, or programmed cell death, is a phenomenon initiated if stress-induced damage is overbearing and repair is not feasible. In other words, it ensures that injured cells do not replicate and pass on genomic damage to progeny cells. Therefore, having the inability to progress with apoptosis after severe damage is disadvantageous for a cell, since replication of lesioned DNA can lead to the immortalization of genomic mutations and potentially tumor formation (57). Indeed, many tumors exhibit defects in proteins important in apoptosis – the p53 protein is a good example, since it functions in promoting apoptosis after stress and is one of the most frequently mutated gene in human tumors (58). On the other hand, the cell must also have proteins that negatively regulate
apoptotic pathways in the event that stress-induced damage is efficiently repaired, and Wip1 is one of these proteins.

p53-dependent apoptosis is well characterized and involves a number of signaling proteins to induce caspase-dependent and mitochondrial apoptotic pathways (reviewed in (58)). Since p53 is a director of apoptosis, Wip1 inhibition of p53 by direct dephosphorylation and through the inactivation of upstream proteins not only results in the weakening of cell cycle checkpoints as discussed above, but also promotes cell survival by suppressing apoptosis (Figure 3). For example, Wip1 suppresses myc-induced apoptosis through inhibition of the ATM-p53 apoptotic pathway and promotes tumorigenesis. Myc-induced lymphoma tumors developed in $PPM1D^+$ mice had elevated levels of apoptosis compared to control mice, and additional deletions of ATM or p53 lowered apoptotic levels similar to those seen in tumors from ATM$^+$ or p53$^+$ mice (2). Thus, Wip1 inactivates ATM signaling to p53 and encourages cell survival when myc expression levels are high.

Wip1 inhibition of apoptosis is also a consequence of the negative feedback exerted on the p38-p53 pathway. Takekawa et al showed that Wip1 over-expressing A549 cells (p53-proficient) have a significant decrease in apoptosis after UVR compared to control cells, and this decrease is dependent on Wip1 phosphatase activity since over-expression of a Wip1 phosphatase dead mutant has no effect (24). Complementary to this study, Schito et al illustrated that the DP thymocyte population (double-positive for CD4 and CD8) and not the DN population (double-negative for CD4 and CD8) from
*PPM1D*<sup>−/−</sup> mice undergo a higher rate of apoptosis (59). The relevance to Wip1 negative feedback on the p38-p53 pathway resides in the fact that active p38 facilitates early thymocyte differentiation, but inactivation of p38 (and subsequent p53 inactivation) is necessary for thymocyte differentiation from DN to DP. Hence, the activation of p38 persists in mice deficient in Wip1, which leads to both a defect in T-cell maturation to DP and higher apoptotic levels in the DP population due to higher quantities of p53 activation (59).

The effect of Wip1 over-expression on Chk2-dependent apoptosis has also been examined. Chk1 and Chk2 function in apoptosis signaling pathways, as both proteins phosphorylate p53 and E2F1, which facilitates both p53-independent and p53-dependent apoptosis (60). Phosphorylation of S215 and T68 sites on Chk2 are important for apoptosis, and phosphorylation of these residues are reduced after stress in Wip1 over-expressing cells compared to control cells (22). As anticipated, over-expression studies in cells show that Wip1 inhibits apoptosis by decreasing phosphorylation of Chk2 on S516 and T68 after IR and that Chk2 and Wip1 interact through the SQ/TQ domain of Chk2 and the N-terminal domain of Wip1 (22,45). Therefore Wip1 inhibits apoptosis through the inhibition of ATM, Chk1, Chk2, and p53 (Figure 3).

1.6 Wip1 inhibits DNA repair.

The integrity of the genome is constantly challenged throughout the life of a cell, and given the considerable variety of DNA lesions, different DNA repair processes exist to reverse each type of DNA damage (35). Modulation of the DNA repair pathways is
critical for subsequent cellular recovery. Since Wip1 reverses cell signaling involved in cell cycle arrest and apoptosis after genotoxic stress, it is likely that Wip1 also functions in regulating DNA repair pathways, although this area remains largely unexplored.

Wip1 has been shown to inhibit base excision repair (BER), which is a DNA repair pathway stimulated by both endogenous and exogenous genotoxic stresses (reviewed (61)). Briefly, in BER there are certain sensor proteins that scan the genome in search of an incorrect base pair, excise the base, recruit other proteins involved in synthesizing new DNA, and finally seal the nick to completely return the DNA to the double helix conformation (61). An example of a BER sensor protein is uracil DNA glycosylase (UNG), which recognizes uracils in the DNA resulting from occurrences such as deamination of cytosines within cyclobutane pyrimidine dimers generated by UVR radiation (61). UNG2, the principle UNG in the cell, is activated in response to UVR by phosphorylation on residues T6 and T126 (25), and phosphorylation of these sites, therefore, is indicative of an increase in BER activity. Like other processes activated after stress, activated BER proteins must be inactivated to return the cell to the homeostatic state, and Wip1 has been shown to participate in BER suppression after UVR. MEFs derived from $PPM1D^+$ mice exhibit an increase in BER compared to control MEFs both in vivo and in vitro (25). This Wip1-induced inhibition of BER is independent of p53, since this effect is seen in Wip1 overexpressing, p53-deficient Saos-2 cells (25).
The proposed mechanism of BER suppression by Wip1 is through dephosphorylation of UNG2 on one of its activating residues, T6. Evidence supporting this mechanism includes the fact that Wip1 interacts with UNG2 in vivo and dephosphorylates UNG2 in vitro (Table 3 and Table 4) (25,49). In addition, over-expression of Wip1 in U2OS cells reduces phosphorylation on T6 of UNG2 after UVR treatment (25). Hence, Wip1 suppresses BER after UVR exposure by directly dephosphorylating UNG2 on T6 (Figure 3).

1.7 Wip1 and aging

In addition to its effect on stress signaling, Wip1 has been shown to play a role in aging in certain cell types by reducing proliferation of stem cells and early progenitor cells (62). This role of Wip1 has been shown in β-cells of the pancreas and in the adult olfactory bulb (63,64). In β-cells of the pancreas, Wong et al show that Wip1 expression decreases with age, which results in heightened p38 activity. Active p38 then increases the expression of Cdkn2a, which reduces cell proliferation and subsequent regeneration of differentiated β-cells (63). On the other hand, Wip1 contributes to the regeneration of new neural cells through a p53-dependent mechanism (64). Deletion of Wip1 in mice resulted in an increase in p53 activity and a significant reduction of the neural progenitor stem cell pool, which was completely reversed by p53 depletion (64). Further analysis revealed that the depletion of the neural progenitor stem cell pool by Wip1 deficiency was specifically due to an inhibition of p53-dependent M-phase cell cycle entry. Therefore, another physiological function of Wip1 is to replenish stem cell pools in
certain tissue types, and the decrease of Wip1 expression in these cell types with time causes an aged phenotype (62).

1.8 Wip1 and senescence

A function for Wip1 in senescence was first implied by the reduced proliferative capacity of mouse embryonic fibroblasts (MEFs) harvested from Wip1-/- compared to the wild type controls (27). In addition to a reduced proliferation rate, Wip1-/- MEFs showed a growth cessation after around thirteen passages whereas wild type MEFs continued to grow for many more passages, which is indicative of premature senescence in Wip1-/- MEFs (27). Indeed, a role for Wip1 in senescence was confirmed by Lee et al (65). These authors show that Wip1 expression is sufficient to overcome premature senescence of human mesenchymal stem cells (hMSCs). These authors also show that premature senescence of hMSCs in culture is caused by activation of p38 and subsequent increasing levels of p16$^{INK4a}$ and Retinoblastoma (Rb) activation, presumably caused by the presence of reactive oxygen species (ROS). Wip1 expression in hMSCs lead to an inhibition of p38 activity and a decrease in p16$^{INK4a}$ levels and extended the life of these cells in culture by overcoming premature senescence (65). This function of Wip1 has direct implications in the development of stem cell therapy for various human diseases, since introduction of Wip1 expression may be a way to overcome the difficulties of culturing and forcing hMSCs differentiation (65,66).
1.9 Summary and project specific aims.

In summary, Wip1 functions to return the cell to homeostasis by directly dephosphorylating several key stress signaling proteins and ultimately inhibiting cell cycle arrest, apoptosis and DNA repair. In the case of Wip1 over-expression, some functions of Wip1, such as the stabilization of MDM2/X and inhibition of p53, are oncogenic. Since Wip1 is over-expressed and amplified in several human cancers, Wip1 is an attractive drug target. As with any drug target, understanding Wip1 molecular functions is important.

Although the current literature sheds light on several details of Wip1 molecular functions, there are some areas that need to be expanded – one of which involves the regulation of Wip1 expression. There are inevitably multiple modes of Wip1 expression regulation since Wip1 is ubiquitously expressed in mouse tissues (27) and has an important role in the response to multiple types of stress (6). An especially intriguing question is the regulation of Wip1 expression during inflammation, given the newly found role of Wip1 to dampen the inflammatory response (46). NF-κB is a well-known family of transcription factors that is activated during inflammation and is constitutively activated in several types of cancer (67). This raises the question, “Does NF-κB regulate Wip1 expression?” This question needs to be addressed since not only would NF-κB-regulated Wip1 expression have implications in inflammation, but it would also suggest a novel mechanism by which Wip1 expression is controlled in tumor cells.
Another gap of knowledge in Wip1 signaling is the role of Wip1 in DNA repair. As explained above, multiple mechanisms by which Wip1 inhibits cell cycle arrest and apoptosis are described in the literature. However, the only evidence of Wip1 inhibition of DNA repair is the inhibition of BER. While this method of repair is important, there are other types of DNA repair that may also be affected by Wip1. For example, it is well-known that Wip1 is upregulated after IR exposure. IR exposure creates multiple types of DNA damage including the most lethal type of damage, the double strand break (DSB). The predominant repair mechanism for DSBs in the cell is Non-Homologous End-Joining (NHEJ). Therefore another important question is, “What is the effect of Wip1 on NHEJ?” It will be especially interesting to determine whether Wip1 affects DNA-PK, one of the main enzymes responsible for NHEJ, since DNA-PK is phosphorylated on multiple residues that have the “SQ” motif Wip1 has been shown to target (68). It makes intuitive sense that Wip1 would have a mechanism for inhibiting NHEJ, since Wip1 attenuates BER and other stress signaling pathways.

Wip1 is currently known to inhibit pro-apoptotic and cell cycle arrest pathways after genotoxic stress; however, little is known about how Wip1 affects other pro-survival stress-activated signaling pathways, such as the NF-κB pathway. Although Chew et al. show that Wip1 inhibits NF-κB signaling during inflammation (46), it is possible that the effects of Wip1 on NF-κB may be different depending on the context. For example, NF-κB is activated after IR exposure and induces the expression of anti-apoptotic genes such as c-IAP1/2 (69-72), and, since Wip1 has been shown to inhibit apoptosis by other mechanisms, it is possible that Wip1 may enhance NF-κB signaling as another way of
inhibiting apoptosis after IR. Additionally, Wip1-/ mice have immune deficiencies (as described above) (27), and the lack of Wip1 complementation of NF-κB may account for this phenotype. In addition to its role in inflammation, NF-κB is known to be constitutively active in many cancers (73,74). Due to the fact that Wip1 has been shown to promote tumorigenesis, Wip1 may enhance NF-κB in the context of some cancer cells. Therefore, since NF-κB signaling is complex and context-dependent, the effects of Wip1 on NF-κB signaling may also be context dependent, and this needs to be evaluated.

1.9.1 Hypothesis

Wip1 is induced by various transcription factors, including NF-κB, after stress and dampens the stress response by targeting key proteins such as NF-κB and DNA-PK in signaling pathways important for survival and DNA repair.

1.9.2 Specific Aims (SA)

SA1. Determine whether the NF-κB transcription factors regulate PPM1D transcription.

SA2. Determine whether Wip1 inhibits DNA-PKcs after genotoxic stress, and identify the mechanism of inhibition.

SA3. Investigate Wip1 activation of the NF-κB pathway after stress.

2.1 Materials

The following is a list of chemicals used in these studies along with the concentrations used: IMD-0354 (Sigma Aldrich, St. Louis, M.O., U.S.A.; Cat. # I3159), 2 µM; recombinant mouse Tumor Necrosis Factor-α (TNF-α) (Sigma-Aldrich, Cat. # T7539), 1 ng/ml; lipopolysaccharide (Sigma-Aldrich, Cat. # L4130), 10 ug/ml (cells) and 10mg/kg (C57B/6 mice); Interleukin-1β (Millipore, Cat. # IL038), 10ng/mL; Actinomycin D (Sigma-Aldrich, Cat. # A9415), 1µg/mL; doxycycline (Clontech, Cat. # 631311), 0.5µg/mL. The pDNA-PKcs (T2609) blocking peptide (GenScript, Cat. # RP19823) was used according to the manufacturer’s protocol.

2.2 Cell culture

MCF-7, HCT-116, BJhTert, DU-145, E1A/Ras MEFs (wild type and Wip1-/-) (2), H1299 and PC-3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2mM glutamine at 37°C in 5% CO₂. H1299 tet-on Wip1 inducible cells were made (by Dmitry Bulavin) similar to a tet-on Wip1 inducible Saos-2 cell line made previously (2). Stable H1299 cell lines were made (by Hyukjin Cha, Ph.D.) by transduction of retroviral vectors (pBabe-empty, pBabe-Wip1, and pBabe-Wip1DA). pBabe-Wip1DA includes the Wip1 open reading from with the mutation 314DA, which was constructed by using the QuikChange® site-directed mutagenesis kit as the manufacture recommended (Stratagene, CA, Cat # 200518).
Primary splenic lymphocytes were harvested from wild type or Wip1-/- (27) C57B/6 mice and cultured following a previously described protocol (27).

2.3 Immunoblotting

At the time of harvest, cells were washed with cold phosphate-buffered saline (PBS), scraped and centrifuged (2000 RPM for 2 minutes) to remove PBS. Nuclear extracts were prepared as previously described (43). For whole cell extract isolation, cell pellets were resuspended in tissue lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, and protease inhibitor mixture) and clarified by centrifugation at 13000 RPM for 10 minutes. The protein concentration of each sample was measured using a Bradford assay (Bio-rad Laboratories, Hercules, C.A., U.S.A.; Cat. # 500-0205) with bovine serum albumin (BSA) standards. Approximately 50 µg of total protein from each sample were separated by SDS-PAGE electrophoresis, transferred to a polyvinylidene difluoride membrane, and individual proteins were then detected as previously described (43). Specific primary antibodies used include p65 (Cat. # sc-372), β-actin (Cat. # sc-47778), and p38 (Cat. # sc-7972) from Santa Cruz Biotechnology, Inc (Santa Cruz, C.A., U.S.A.). Wip1 (Bethyl Laboratories, Montgomery, T.X., U.S.A.; Cat. # A300-664A), phospho-p38 (T180/Y182) (Cell Signaling Technology, Danvers, M.A., U.S.A.; Cat. # 9216), IκBα (Cell Signaling Technology, Cat. # 4814), pDNA-PKcs (T2609) (Biolegend, Cat. # 612901) were also used.
2.4 siRNA and plasmid transfections

RELA (p65) and GFP targeted siRNA were purchased from Dharmacon, Inc. (Chicago, I.L., U.S.A.; Cat. # J-003533-06 and P-002048-01-20, respectively). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, C.A., U.S.A.; Cat. # 11668027) following the manufacturer’s protocol with 100 pmole of siRNA (and 2 µg of plasmid DNA for cotransfections) per 60 mm tissue culture plate.

2.5 Quantitative Real Time PCR

At the time of harvest, cells or organ tissues were homogenized in Trizol (Invitrogen, Cat. # 15596018), and RNA was isolated following the manufacturer’s protocol. RNA cleanup and concentration was performed using the RNeasy MinElute Cleanup Kit (Qiagen, Valencia C.A., U.S.A.; Cat. # 74204). 500 ng of RNA from each sample was used in a single qRT-PCR reaction. Brilliant® II QRT-PCR Master Mix, 1-step (Stratagene, La Jolla, C.A., U.S.A.; Cat. # 600809) was used with the specified taqman probe/primer mix from Applied Biosystems, Inc. (Foster City, C.A., U.S.A) including human PPM1D (Cat. # NM_003620.2), mouse Ppm1d (Cat. #

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<tr>
<td>FP1</td>
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<tr>
<td>FP2</td>
<td>ACCGAGACTGTGCAGCTG ACGGTTAAGTGCTT</td>
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<tr>
<td>RP1</td>
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<td>RPC1</td>
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<tr>
<td>FPC1</td>
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</tr>
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</table>

**Table 5**

Primers used for the cloning of “pWip1-luc,” “pWip1_ΔkB-luc,” “pWip1_ΔC/EBPα-luc,” and “pWip1_ΔkB-C/EBPα-luc.” (Eurofins MWG Operon, Huntsville, AL, U.S.A.)
NM_016910.2), human GAPDH (Cat. # 4326317E), and mouse GAPDH (Cat. # 4352339E). IκBα primers used were as follows: forward primer:

TCGCTCTTTGGAAATGTGG, reverse primer: TCATAGGGCAGCTCATCCTC.

qRT-PCR reactions were run with the Bio-rad iCycler (Bio-rad Laboratories) and the following parameters: 1) 95°C for 3 min (1X), 2) 95°C for 15 sec followed by 60°C for 1 min (50X), 3) 95°C for 1 min (1X), and 4) 60°C for 20 sec (50X). Data analysis was performed using the iCycler IQ version 3.1 software (Bio-rad Laboratories).

### 2.6 Plasmid construction and luciferase assays

For the construction of the four PPM1D reporter plasmids used, “pWip1-luc,” “pWip1_ΔκB-luc,” “pWip1_ΔC/EBPα-luc,” and “pWip1_ΔκB-C/EBPα-luc,” the appropriate sequences were cloned into the Rapid Reporter® pRR-High vector (Active Motif, Carlsbad, CA, U.S.A.; Cat. # 33003). Cloning of the 855 bp PPM1D promoter sequence was based on previously described cloning of this region (36). Briefly, a section of the PPM1D promoter region including the 855 bp sequence between AvrII and BamHI restriction sites was amplified from genomic DNA isolated from MCF-7 cells using primers FP1 and RP1 (see Table 5). The κB site was deleted by using two different

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>PPM1D-FP1</td>
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</tr>
<tr>
<td>PPM1D-RP1</td>
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</tr>
<tr>
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</tr>
<tr>
<td>NFKBIA-RP1</td>
<td>TCAGGCTCGGGGAATTGCC</td>
</tr>
</tbody>
</table>

Table 6

Primers used for the ChIP analysis of the PPM1D and NFKBIA promoter regions. Primers obtained from Eurofins MWG Operon.
sets of primers that, after amplification, result in two overlapping fragments that have the κB site deleted (FP1 and RP2, FP2 and RP1; see Table 5). The two overlapping (κB site deleted) fragments were then annealed by overlap extension in a PCR reaction. The fragments of the PPM1D promoter with the C/EBPα site deleted (“pWip1_ΔC/EBPα-luc”) and with both the κB site and C/EBPα site deleted (“pWip1_ΔκB-C/EBPα-luc”) were made in the same manner. The primer sets used for pWip1_ΔC/EBPα-luc are FP1/RP1 and FPC1/RP2, and those used for pWip1_ΔκB-C/EBPα-luc are FP1/R2 and FPC1/RP1 (Table 5). All of the four amplicons were digested with AvrII and BamHI enzymes and cloned into the SpeI and BglII sites of the RapidReporter® pRR-High vector to generate pWip1-luc and pWip1_ΔκB-luc, respectively. Correct plasmid construction was confirmed by sequencing.

The RapidReporter® pRR-High-NF-κB and RapidReporter® pRR-High-EF1a (control) vectors were purchased from Active Motif (Cat. # 33009) and used according to the manufacturer’s protocol. The IκBα promoter luciferase construct was a gift from Dr. Paul J. Chiao (The University of Texas M. D. Anderson Cancer Center, Houston, T.X.). Luciferase activity of cells transfected with the luciferase reporter vectors was measured using the RapidReporter® Gaussia Luciferase Assay (Active Motif, Cat. # 33001) according to the manufacturer’s protocol.

2.7 Chromatin Immunoprecipitation Assays (ChIP)

ChIP analysis was run on three 100 cm plates per treatment of MCF-7 cells using the ChIP-IT™ Express Enzymatic kit (Active Motif, Cat. # 53009) according to the
manufacturer’s protocol. A ChIP grade NF-κB p65 antibody was used and purchased from Abcam, Inc. (Cambridge, M.A., U.S.A.; Cat. # ab7970). The IgG negative control antibody and the forward and reverse negative control primers used were from the ChIP-IT™ Control Kit – Human (Active Motif, Cat. # 53010). The sequence of the NFκBIA (75) and PPM1D primers are listed in Table 6.

2.8 Ionizing Radiation

Cells were exposed to ionizing radiation using a γ-iradiator (cesium-37 source). Unless otherwise indicated, cells were exposed to 5 Gy.
Chapter 3 publication reference

The following chapter (Chapter 3: “Nuclear Factor-kappa Beta (NF-κB) is a novel positive transcriptional regulator of the oncogenic Wip1 phosphatase”) is in press in The Journal of Biological Chemistry and will be published in February 2010. Please see below for the reference.

Chapter 3. Nuclear Factor-kappa Beta (NF-κB) is a novel positive transcriptional regulator of the oncogenic Wip1 phosphatase.

3.1 Introduction

The family of transcription factors named NF-κB (Nuclear Factor-Kappa Beta) has been well-studied and plays an important role in inflammation, in cancer, and in the cellular response to a variety of stresses such as genotoxic stress (67). The mechanisms of NF-κB activation have been reviewed and are dependent on the cell type and stimulus (67,76). Briefly, the NF-κB subunits are held in the cytoplasm by binding to the inhibitor protein, IκB (Inhibitor of NF-κB). Upon stimulation, IκB is phosphorylated by the IKK (Inhibitor of NF-κB Kinase) complex and then ubiquitinated and targeted for proteasomal-mediated degradation. Once IκB is degraded, the NF-κB subunits translocate into the nucleus, are further regulated by post-translational modifications, and are able to either promote or repress transcription by directly binding to κB sites in the promoter regions of target genes (67).

NF-κB signaling has gained significant attention in cancer research, especially since there is increasingly more evidence linking inflammation to the initiation, promotion and progression of cancer through NF-κB activation (73,77,78). NF-κB can be activated in normal tissue by carcinogens, DNA damaging agents, or pro-inflammatory cytokines secreted by stimulated leukocytes during chronic inflammation such as that associated with bacterial or viral infection. Additionally, NF-κB activation occurs in tumor cells either by leukocytes surrounding the tumor microenvironment or
acquired constitutive NF-κB activation. Once activated, the NF-κB transcription factors increase the expression of genes important for cell growth, inhibition of apoptosis, metastasis, and angiogenesis – all of which promote cancer progression at various levels (78). There are many examples of the pro-survival features of NF-κB, e.g. an activator for this pathway substantially reduced radiation-induced killing in mice at doses which are normally lethal (79).

In this chapter, the oncogenic Wip1 phosphatase is identified as a positively regulated target gene of NF-κB. Wip1 expression, at both mRNA and protein levels, is enhanced or repressed in cells that have stimulated or inhibited NF-κB activity, respectively. Furthermore, NF-κB regulates PPM1D transcription by directly binding to the PPM1D promoter region. Finally, Wip1 expression induced in a NF-κB-dependent manner in LPS-stimulated mouse splenic B-cells facilitates proliferation. These data have important implications for Wip1 oncogene regulation by NF-κB in human cancers.

3.2 Results

3.2.1 The PPM1D promoter region includes an evolutionarily conserved κB site.

In a search for transcription factors that regulate PPM1D expression, analysis of the 1000 base pairs immediately upstream of the PPM1D translational start site revealed a κB site, which is a conserved binding sequence for the NF-κB transcription factors (Figure 4A). Further analysis of the promoter region in several organisms among the evolutionary hierarchy ranging from humans to zebrafish showed conservation of this κB site (Figure 4B). Both the sequence and the position (relative to the translational start
The *PPM1D* promoter region includes an evolutionarily conserved κB site. A) A conserved κB site was found at position -793bp in the *PPM1D* promoter region using the Alibaba 2.1 software (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). B) The κB site is highly homologous in sequence and in position with respect to the translational start site among the various organisms. The κB site are highly homologous among the different species, implicating evolutionary importance of the κB site in the *PPM1D* promoter region and, most likely, *PPM1D* transcriptional regulation.
3.2.2 Inhibition of NF-κB decreases Wip1 expression.

The presence of a κB site implies that the NF-κB transcription factors regulate *PPM1D* transcription; therefore, we next sought to determine whether alteration of NF-κB activity affected Wip1 expression. In order to inhibit NF-κB activity, we used the

**Figure 5**

**Inhibition of NF-κB decreases Wip1 expression.** A) Nuclear extracts were immunoblotted for p65 and β-actin (loading control), B) Wip1 mRNA levels were quantified, or C) whole cell lysates were immunoblotted for
chemical inhibitor of IKKβ, IMD-0354 (80,81), which ultimately inhibits IκBα degradation and translocation of the NF-κB subunits into the nucleus. After incubation of IMD-0354 for various time periods with MCF-7 breast cancer cells, which have about a threefold increase in Wip1 expression (9), nuclear extracts were immunoblotted for the NF-κB subunit p65 to confirm that IMD-0354 effectively inhibits NF-κB in these cells. As shown in Figure 5A, nuclear levels of p65 decreased in cells treated with IMD-0354 compared to the DMSO controls in a time dependent manner, which indicates a reduction in p65 activity. qRT-PCR analysis of similarly treated cells revealed that Wip1 mRNA levels decreased in IMD-0354 treated cells with time compared to the controls (Figure 5B). The effect of IMD-0354 on Wip1 expression is not solely at the mRNA level, since immunoblotting of whole cell lysates from these cells for Wip1 revealed that IMD-0354 treatment also decreased total Wip1 protein (Figure 5C).
As an alternative method of NF-κB inhibition, MCF-7 cells were transfected with siRNA targeted to p65. As with inhibition of NF-κB by IMD-0354, qRT-PCR and

**Figure 6**

**Activation of NF-κB increases Wip1 expression.** Wip1 mRNA levels were quantified by qRT-PCR and normalized to GAPDH after NF-κB activation. A) MCF-7 cells were incubated with either IMD-0354 alone, TNFα alone, or TNFα with pretreatment of IMD-0354 for the indicated time periods. B) Primary splenic lymphocytes from wild type mice were incubated with LPS with or without pretreatment of IMD-0354 for the indicated time periods. Dotted lines represent the value of the untreated control samples, which are set to one.
immunoblotting analysis demonstrated that knockdown of p65 expression also resulted in a reduction of Wip1 mRNA levels (Figure 5D) and total protein (Figure 5E), respectively, by about 50%. We would expect there to be more dramatic changes in Wip1 expression if the transfection efficiency of the siRNA in these cells was higher. Taken together, these findings show that inhibition of p65 activity in MCF-7 cells inhibits the expression of Wip1 at both the protein and mRNA levels, showing that p65 is a positive regulator of Wip1 expression.

3.2.3 Activation of NF-κB increases Wip1 expression.

Since reducing NF-κB activity decreased Wip1 expression, enhancing NF-κB activity should increase Wip1 expression. In order to test this, TNFα was incubated with MCF-7 cells since it is a well characterized activator of NF-κB (82,83). Two and four hours after incubation of TNFα, qRT-PCR analysis shows that Wip1 mRNA levels dramatically increase (Figure 6A). Furthermore, pre-incubation with IMD-0354 severely inhibits this increase (Figure 6A), indicating that TNFα-stimulated Wip1 induction is NF-κB dependent.

To supplement the above results, we also measured Wip1 mRNA levels in LPS-stimulated primary splenic lymphocytes harvested from wild type mice. LPS-stimulated B-cell proliferation is a process in which NF-κB activation is required (84-86). Additionally, Wip1 has been shown to be important in LPS-stimulated B-cell proliferation, particularly at 24 and 48 hours after LPS addition (27), suggesting that Wip1 expression might be modulated in this system. As expected, Wip1 mRNA levels
Figure 7

**p65 activity regulates PPM1D promoter activity and directly binds to the PPM1D promoter region.** A) Schematic of the construction of the PPM1D luciferase reporter vectors. “pWip1-luc” includes the complete 855 base pair sequence directly upstream from the PPM1D translational start site, and “pWip1_ΔκB-luc” has the κB site deleted. B) PPM1D promoter activity assay. MCF-7 cells were transfected with either pWip1-luc or pWip1_ΔκB-luc alone or cotransfected with p65 siRNA. Luciferase activity was measured 48 hours later. C) ChIP analysis of NF-κB promoter binding. MCF-7 cells were either untreated, treated with TNFα alone, or treated with TNFα after a 5 hour pre-treatment with IMD-0354. The cells were harvested for ChIP assays using either IgG (negative
greatly increase at 24 and 48 hours after LPS stimulation (Figure 6B). Similar to the TNFα results above, pre-incubation of IMD-0354 with the lymphocytes inhibited the LPS-stimulated increase in Wip1 mRNA (Figure 6B), indicating that the regulation of Wip1 expression is dependent on NF-κB activation. Taken together, these data show that Wip1 expression increases in response to LPS and TNFα stimulation through NF-κB activation in splenic B-cells and MCF-7 cells, respectively.

3.2.4 NF-κB regulates PPM1D promoter activity and binds to the PPM1D promoter region.

Thus far, we have shown that NF-κB regulates Wip1 expression. Since the mechanism of action of the NF-κB family members is through transcriptional modulation of target genes (67), we wanted to test whether NF-κB directly regulates PPM1D transcription. First, PPM1D promoter activity was measured with luciferase reporter constructs in cells with and without altered NF-κB activity. Figure 7A highlights the design of the two constructs – they are identical with the exception of the deletion of the κB site in one of the vectors. As shown in Figure 7B, the activity of the wild type promoter (“pWip1-luc”) decreases by more than 60% in cells with depleted p65 expression by siRNA treatment. On the other hand, the basal activity of the κB site-deleted PPM1D promoter (“pWip1_ΔκB-luc”) is substantially reduced compared to the
wild type promoter (Figure 7B), and depletion of p65 by siRNA does not significantly alter the activity of the mutated PPM1D promoter. These data indicate that (i) full basal PPM1D promoter activity requires the conserved κB site and (ii) the NF-κB subunit p65 regulates basal PPM1D transcription in MCF-7 cells. Similar results were seen after ionizing radiation exposure (IR, Figure 8A and B) and TNFα stimulation (Figure 8C), which are also NF-κB activators. As seen in Figure 8A and B, wild type PPM1D

![Figure 8](image)

**Figure 8**

**NF-κB regulates PPM1D promoter activity after ionizing radiation and TNFα stimulation.** HCT116 cells were transfected with either A) pWip1-luc or B) pWip1_ΔκB-luc. The cells were then treated with either IMD-0354 alone, a 5 Gy exposure of ionizing radiation, or both. Luciferase activity was measured two hours later. C) MCF-7 cells were transfected with either pWip1-luc or pWip1_ΔκB-luc and treated with either IMD-0354 alone, TNFα alone, or both. Luciferase activity was measured 24 hours later.

promoter activity increases with IR, which is inhibited by IMD-0354, where as the
mutant PPM1D promoter activity increases (most probably due to p53 activity (36)) but is not affected by IMD-0354. Additionally, wild type PPM1D promoter activity increases with TNFα, which is inhibited by IMD-0354, where as the mutant PPM1D promoter is not activated by TNFα (Figure 8C). These results indicate that Wip1 expression is regulated by NF-κB in response to TNFα and (partially, along with p53) to IR stimuli.

In order to test the possibility that the NF-κB subunits directly regulate PPM1D transcription by binding to the promoter region, ChIP assays were performed. The p65 subunit constitutively binds to the PPM1D promoter region in MCF-7 cells, indicated by the amplification of the promoter region that encompasses the κB site from chromatin samples immunoprecipitated with a p65 antibody but not IgG serum (Figure 7C). After a three hour treatment with TNFα, the amount of p65 bound to the PPM1D promoter region increases compared to basal levels (Figure 7C), which is similar to p65 binding to the promoter of NFKBIA, the gene encoding IκBα (positive control). The reason why there appears to be a larger effect of TNFα-induced p65 binding to the NFKBIA than to the PPM1D promoter is unclear, but this may be due to the higher amount of basal p65 binding to the PPM1D and not the NFKBIA promoter region. Furthermore, inhibition of TNFα-induced NF-κB activation by pre-treatment with IMD-0354 reduces the amount of p65 subunits bound to the PPM1D promoter region to basal levels (Figure 7C). These results demonstrate that the p65 NF-κB subunit directly regulates PPM1D transcription basally and after TNFα stimulation in MCF-7 breast cancer cells.
3.2.5 Wip1 expression in LPS-stimulated splenic B-cells is inversely correlated with p38 activation and promotes proliferation.

We next sought to determine the function of NF-κB-dependent Wip1 expression in LPS-stimulated B-cells, since Wip1-/− LPS-stimulated B-cells show reduced proliferation (27). In doing so, we focused on determining whether p38 may play a role in the proliferation deficiency seen in Wip1-/− B-cells since p38 is a known Wip1 target (1,8,16,24,38,49) and plays a major role in cytokine production and B-cell proliferation (87,88). p38 activation at early time points after LPS stimulation is known to positively regulate B-cell proliferation (87,88), yet the effects of p38 activity at later time points when proliferation is high and Wip1 expression is increased (24 and 48 hours) have not been extensively studied. Therefore, we measured the levels of active p38 at various times after LPS stimulation. As seen in Figure 9A, phospho-p38 (T180/Y182, active p38) increases two hours after LPS stimulation, as expected, and then decreases at the 24 and 48 hour time points. Wip1 expression, on the other hand, is only increased at 24 and 48 hours after LPS addition and not at two hours (Figure 9A). Furthermore, lymphocytes isolated from Wip1-/− mice show heightened phospho-p38 levels 24 and 48 hours after LPS stimulation (Figure 9B). These results demonstrate an inverse correlation of p38 activation and Wip1 expression, suggesting that a possible role for Wip1 is to inactivate p38 at later time points of LPS stimulation in B-cells.

The fact that p38 activity decreases 24 and 48 hours after LPS treatment suggests that p38 needs to be inactivated at these late time points. Given that p38 plays a major role in cytokine production, which is crucial for proliferation, a reduction of LPS-
Figure 9

Wip1 expression in LPS-stimulated splenic B-cells inversely correlates with p38 activity.  A) Primary splenic lymphocytes from wild type mice were incubated with LPS for the indicated time periods. Whole cell lysates were subject to immunoblot analysis, and the fold change in Wip1 mRNA levels (normalized to GAPDH) was measured by qRT-PCR. B) Primary splenic lymphocytes from Wip1-/- mice were stimulated with LPS (or not, “-”), and whole cell lysates were subject to immunoblot analysis. C) Proliferation was measured in LPS-stimulated wild type lymphocytes
stimulated B-cell proliferation by inhibition of p38 would be expected (87,88). However, whether inhibition of p38 at later times after LPS stimulation affects B-cell proliferation is unknown. Proliferation (measured at 48 hours after LPS stimulation) is decreased when the p38 inhibitor SB203580 is added at 8 hours after LPS stimulation, but it is not significantly altered when p38 is inhibited at 24 hours post-LPS stimulation (Figure 9C). These results indicate that p38 activity is low and plays a limited role in proliferation at late time points after LPS stimulation.
If NF-κB induces Wip1 expression, and Wip1 inhibits p38, then inhibition of NF-κB should result in increased levels of active p38. To determine whether p38 activity (like Wip1 expression) is affected by inhibition of NF-κB, LPS-stimulated lymphocytes were either untreated or pre-treated with IMD-0354. Pre-treatment of IMD-0354

**Figure 10**

**NF-κB-induced Wip1 promotes LPS-stimulated B-cell proliferation.** A) The relative proliferation was calculated for primary splenic lymphocytes at the indicated times of LPS incubation (“wild type,” lymphocytes harvested from wild type mice; “wild type, IMD-0354,” lymphocytes harvested from wild type mice treated with IMD-0354; “Wip1-/-,” lymphocytes harvested from Wip1-/- mice). B) Proliferation was measured at 24 and 48 hours after LPS stimulation in wild type splenic lymphocytes without IMD-0354 (“-”) or with IMD-0354 added at the indicated time periods after LPS addition.
enhances p38 activation levels at 24 and 48 hours (Figure 9D). These data indicate that NF-κB decreases p38 activity at later times after LPS addition, presumably through Wip1.

To eliminate the possibility that IMD-0354 pre-treatment indirectly decreases Wip1 expression by affecting signaling at early time points, Wip1 expression was measured when IMD-0354 was added at various times after LPS stimulation. As seen in Figure 9E, Wip1 mRNA levels still decrease when IMD-0354 is added 8, 15, and 24 hours after LPS stimulation, which indicates that the effect of IMD-0354 on LPS-induced Wip1 expression is not due to inhibition of signaling at early time points.

A functional role of NF-κB-induced Wip1 expression is to facilitate LPS-stimulated B-cell proliferation, since Wip1-/- B-cells show reduced proliferation compared to wild type controls (Figure 10A and (27)). Since NF-κB induces Wip1 expression, which then regulates LPS-stimulated proliferation, then inhibition of NF-κB by IMD-0354 should also reduce proliferation. Pre-incubation of IMD-0354 with the wild type splenic lymphocytes showed a significant deficiency in LPS-stimulated proliferation at 48 hours (Figure 10A). Again, to rule out the possibility that IMD-0354 pre-incubation affects proliferation by regulating early signaling and not later Wip1 induction, incubation of the B-cells with IMD-0354 at various times after LPS addition also dramatically reduced proliferation at 24 and 48 hours compared to the untreated controls (Figure 10B). In fact, addition of IMD-0354 fifteen hours after LPS addition (Figure 10B) results in a reduced proliferation level at 48 hours similar to that of the Wip1-/- lymphocytes (Figure 10A). However, the fact that there is a more dramatic
decrease in proliferation when IMD-0354 is pre-incubated in wild type lymphocytes indicates that there are probably other NF-κB targets that play a role in this process. Therefore, although Wip1 may not be sufficient for LPS-stimulated lymphocyte proliferation, it is necessary for full proliferation. Taken together, these data show that NF-κB-dependent Wip1 expression facilitates LPS-stimulated proliferation in lymphocytes possibly (at least in part) through a p38-dependent mechanism.

3.3 Discussion

These results demonstrate that NF-κB regulates Wip1 expression by directly binding to the *PPM1D* promoter region to enhance transcription and that NF-κB-dependent Wip1 expression facilitates proliferation in stimulated B-cells. Additionally, NF-κB driven Wip1 expression in LPS-stimulated B-cells promotes proliferation by dampening p38 activity at late time points after stimulation. However, the entire scope of NF-κB and Wip1 involvement in B-cell proliferation most likely exceeds that of the single mechanism presented in this chapter. Nevertheless, these findings have multiple implications in the role of Wip1 in the progression and treatment of cancer as well as inflammation.

Enhancing the expression of Wip1 may be an additional way in which NF-κB activity contributes to the growth of cancer cells. The fact that Wip1 deletion stunted LPS-stimulated proliferation demonstrates a proliferative function for NF-κB-dependent Wip1 induction in lymphocytes, but a similar mechanism may also exist in cancer cells. Evidence for this lies in the fact that we show NF-κB as a direct enhancer of Wip1
expression in human breast cancer cells, and others have shown that Wip1 enhances proliferation and inhibits apoptosis in these cells (89,90). Inhibitors of NF-κB have been suggested as chemotherapeutic agents for cancer patients (73,78,91,92). Inhibition of Wip1 expression could be an added benefit of inhibiting NF-κB in this setting, which may be especially important in patients whose tumors have PPM1D amplified and Wip1 over-expressed.

It should be noted, however, that the negative feedback loop between Wip1 and NF-κB may not occur in some cancer cells since Wip1 regulation of NF-κB may be context-dependent. NF-κB activity regulation, including p65 phosphorylation, is extremely complex and has been shown to be dependent on the type of stimulus and tissue (67,76,93). Additionally, the biological consequences of NF-κB activation depend on tissue and stimulus type. For example, constitutive NF-κB activity in the background of inflammation promotes tumorigenesis in some tissues (78), where as NF-κB activity inhibits tumorigenesis in some types of tissues, such as the skin (94). Therefore, there are probably several effects of NF-κB-induced Wip1 on tumorigenesis depending on the context. As previously shown, Wip1 may inhibit tumor suppressors such as p53 or p16/p19 (1,3,6). Wip1 may inhibit NF-κB in some tissues, or Wip1 may not inhibit constitutive NF-κB activity in certain types of cancer cells to promote tumorigenesis.

Not only could inhibition of NF-κB activity, and therefore Wip1 expression, be used for the killing of tumor cells, but it may possibly be used to sensitize tumor cells to radiotherapy. NF-κB activation is a means of adaptive radioresistance in cancer cells.
(95-97), and inhibition of NF-κB has been shown to radiosensitize a number of human cancers (98-102). Enhancing Wip1 expression in tumor cells (either basally or after radiation exposure) may be one way in which NF-κB facilitates tumor cell survival after radiotherapy, since Wip1 is a known inhibitor of many pathways of the DNA damage response that lead to apoptosis, cell cycle arrest, and DNA repair (6).

Our data also provide additional evidence for a role for Wip1 in inflammation. The first evidence of this came from the analysis of the phenotype of the Wip1−/− mouse, which revealed that these mice have compromised immunocompetency due to the presence of abnormal lymphoid structure, increased susceptibility to ulcerated skin lesions and pathogens, and defective T- and B- cell responses (7) (as we also demonstrate here). NF-κB is highly activated by inflammatory signals and has a crucial role in inflammatory processes (67,86), and, as mentioned above, Chew et al recently showed that Wip1 can dampen the inflammatory process by negatively regulating NF-κB signaling by directly dephosphorylating p65 (46). These data, together with the data presented here, provide a mechanism for the role for Wip1 in inflammation—NF-κB is activated by inflammatory signals and enhances the expression of Wip1, which then inhibits NF-κB signaling through a negative feedback loop to presumably turn off the inflammatory process and return the cell to homeostasis.

NF-κB regulation of Wip1 expression adds to the complex network of stress signaling pathways including several feedback loops (Figure 11), which if improperly regulated can contribute to tumorigenesis. The function of both NF-κB and Wip1 proteins is to negate stress signaling and facilitate returning the cell to homeostasis. NF-
κB inhibits the pro-apoptotic signaling of p53 (103,104) and increases anti-apoptotic gene expression (67), which result in cell survival. Wip1 signaling has similar effects, since Wip1 inhibits apoptosis and cell cycle arrest by inactivating several key stress signaling proteins (1). Another common theme of NF-κB and Wip1 signaling is that both enhance MDM2 expression – NF-κB enhances MDM2 mRNA levels and Wip1 dephosphorylates and stabilizes MDM2 protein. By stabilizing MDM2, NF-κB and Wip1 negatively regulate p53-dependent apoptotic signaling (1,103). Additionally, Chew et al recently showed that Wip1 can negatively regulate NF-κB signaling by directly dephosphorylating the p65

**Figure 11**

**Schematic of Wip1 signaling.** *Contributions from the studies presented in this chapter.*
subunit on S536 (46). As outlined in Figure 11, there are several feedback loops that exist in this complex stress signaling network. p53 directly upregulates Wip1, which then inhibits p53. NF-κB directly upregulates Wip1, which then inhibits or enhances NF-κB signaling (Figure 11). p53 enhances MDM2 expression, which then inhibits p53 as the stress response subsides (56). Thus, our results provide a link between multiple stress signaling pathways that return the cell to homeostasis, which may promote tumorigenesis when these pathways are not properly regulated.

In conclusion, we have demonstrated NF-κB as a direct transcriptional enhancer of Wip1 expression. These data show an additional mechanism by which NF-κB may promote tumorigenesis and tumor cell growth, support the use of NF-κB inhibitors for chemotherapeutic and radiosensitizing purposes in the treatment of various human cancers, and further define Wip1 function in inflammation. Although we clearly show that NF-κB constitutively activates Wip1 transcription in breast cancer cells, more studies need to be done to further develop the downstream mechanisms of NF-κB-induced Wip1 expression in the context of cancer cells.
Chapter 4. NF-κB regulation of Wip1 is complex and dependent on stimulus and cell type.

4.1 Introduction

There are certain shared aspects of NF-κB signaling, which involve the IKK complex and the IκB inhibitor proteins. After a certain stimulus, the IKK complex is activated, IκB is degraded, and the NF-κB subunits translocate to the nucleus where they are further regulated by post-translational modifications and ultimately regulate the transcription of target genes (67). However, the regulation of NF-κB signaling is complex and depends on the stimulus and tissue type. There are several points in the shared pathway of NF-κB activation for stimulus-dependent regulation. First, the mechanism of IKK activation may be different. For example, IKK activation in TNF receptor signaling depends on the TRADD, TRAF2, and RIP proteins, whereas IKK is activated in a MYD88-dependent manner in Toll/IL-1 receptor signaling (76).

Additionally, once the NF-κB subunits translocate to the nucleus, the specific post-translational modifications of these subunits are dependent on the stimulus. For example, the p65 NF-κB subunit is phosphorylated on S276 by the mitogen-and stress-activated protein kinase-1 (MSK1) after TNFα stimulation, whereas IKKα phosphorylates S536 after lymphotoxin-β stimulation (93). Thus, the mechanism of NF-κB activation depends on the cellular context.

The disparity of NF-κB signaling ultimately affects the expression of target genes; therefore the transcriptional regulation of different NF-κB target genes also
depends on the cell context (67,76,93). For example, phosphorylation of different sites on the p65 subunit induced by different stimuli affects the expression of certain NF-κB gene targets (67). Additionally, the chromatin structure at the location of some NF-κB target genes may be in a closed conformation and require histone acetyltransferase (HAT) recruitment and activity for transcription initiation, whereas other sites may be in an open conformation and more easily accessible to the NF-κB subunits (105).

Another reason for NF-κB regulation of different genes after different stimuli is that the NF-κB subunits coordinate with different coactivators/corepressors to activate/repress transcription, and the formation of these complexes is stimulus-dependent (67). One example of a protein that coordinates with NF-κB is the CAAT/enhancer binding protein (C/EBP) (106,107). NF-κB and C/EBP are both activated by cytokine stimulation and have been shown to cooperate to regulate the transcription of target genes such as Interleukin-8 (IL-8) and Interleukin-6 (IL-6) (106,108). Cooperation between NF-κB and C/EBP is thought to be complex and dependent on the levels of both transcription factors and the physical association between the two factors (106,107,109). For example, the regulation of IL-8 by C/EBP alone is weak; however, in the presence of high levels of nuclear NF-κB, C/EBP regulation of IL-8 transcription is strong (106). Not only does C/EBP and NF-κB coordinate to induce the transcription of target genes, but C/EBP has also been shown to be required in the NF-κB-dependent repression of certain genes such as the HIV-1 gene specifically in
**Figure 12**

Wip1 mRNA levels are reduced and p38 is activated at early times after LPS stimulation. A) Wip1 mRNA levels were measured from splenic lymphocytes from wild type mice two and four hours after LPS stimulation. B) phospho-p38 (“pp38 (T180/Y182)”) and total p38 protein levels were measured in wild type splenic lymphocytes two, four, twenty-four, and forty-eight hours after LPS stimulation.

The results in this section show that in some contexts, NF-κB may negatively regulate Wip1 expression. Wip1 mRNA levels were reduced at early times after LPS stimulation in splenic lymphocytes and in the lung and liver tissues of LPS challenged mice. Furthermore, Wip1 expression is reduced after IL-1β stimulation, which is an NF-κB activator. The transcription factors responsible for the IL-1β-dependent reduction of brain derived cells (110). Therefore, coactivators or corepressors, such as C/EBP, may be partially responsible for the context-dependence of NF-κB regulation of target genes.
Wip1 expression depends on the time of stimulation. p65 and C/EBPα coordinate to inhibit PPM1D transcription after IL-1β stimulation. p53 also regulates PPM1D transcription during IL-1β signaling, but only at certain times after stimulation. These results show the complexity of NF-κB regulation of Wip1 expression.

4.2 Results

4.2.1 Wip1 expression is reduced at early times after LPS stimulation.

The results from Chapter 3 demonstrate NF-κB as a positive regulator of Wip1; however, regulation of Wip1 by NF-κB appears to be dependent on context. The first clue of the complexity of NF-κB-modulated Wip1 expression came from the analysis of Wip1 expression at early time points after LPS stimulation in primary mouse splenic B-cells. Wip1 mRNA levels were measured at two and four hours after LPS stimulation in mouse splenic lymphocytes, and the fold change in Wip1 mRNA levels (normalized to basal levels) were graphed (Figure 12A). As seen in Figure 12A, Wip1 mRNA levels as measured by qRT-PCR only slightly decreased at two hours, but then dramatically decreased at four hours after LPS stimulation. This is in direct contrast to the effect of LPS on Wip1 expression at later times after stimulation such as 24 and 48 hours, during which Wip1 expression is significantly increased (Figure 9A). Interestingly, p38 activity is increased at the two and four hour time points after LPS stimulation when Wip1 expression levels are reduced (Figure 12A). Therefore, these results are consistent with the idea that p38 activity is inversely correlated with Wip1 expression in primary splenic B-cells at all of the times (or, at least those measured in these studies) after LPS
stimulation, even though there is opposing regulation of both Wip1 expression and p38 activity at early verses late times.

4.2.2 Wip1 expression is reduced in the lungs and liver after LPS administration in mice.

Like primary splenic B-cells, the response of certain tissues in mice to LPS administered via intraperitoneal injection (i.p.) involves activation of NF-κB signaling (111). The degree of NF-κB activation, however, is tissue specific. Blackwell et al use a mouse model in which all tissues express a NF-κB-dependent promoter that controls the expression of a luciferase reporter gene to measure the levels of NF-κB activity in organs of mice challenged with an LPS injection. Certain organs such as the liver and the lungs of these mice showed high LPS-driven NF-κB activation (111). To determine whether Wip1 expression is modulated by NF-κB in vivo, Wip1 mRNA levels harvested from the lungs and liver of mice two hours after an LPS i.p. injection were measured, since these tissues

Figure 13

Wip1 mRNA levels are reduced in the liver and lungs after LPS administration. Wip1 mRNA levels were measured from lung and liver tissue from mice two hours after LPS injection (by intraperitoneal (i.p.)).
have high NF-κB activation in response to LPS. Based on the results described in Chapter 3, an increase in Wip1 mRNA levels was expected, but, in fact, Wip1 mRNA levels in both tissues significantly decreased in response to LPS (Figure 13). These results, taken together with the results above from section 3.4.1, suggest that NF-κB has a negative effect on Wip1 expression at early times after LPS stimulation. Although, Wip1 expression could be negatively affected by another transcription factor(s) activated by LPS at the early time points since LPS signaling is complex and leads to the activation of multiple transcription factors such as the MAPKs (112).

4.2.3 Interleukin-1β (IL-1β) stimulation reduces Wip1 protein levels in MCF-7 cells.

Thus far, the above results show a disparity in the regulation of Wip1 expression by two different NF-κB activators, TNFα and LPS. TNFα enhances PPM1D transcription (Figure 6A). Late LPS signaling enhances Wip1 expression (Figure 6A), where as early LPS signaling decreases Wip1 expression (Figure 12A). However, NF-κB is activated by several different types of stimuli, and another well-described NF-κB activator is Interleukin-1β (IL-1β) (67). Therefore, whether IL-1β stimulation had an effect on Wip1 expression was tested. IL-1β was added to the media of MCF-7 cells, and
Figure 15

**p65 regulates Wip1 protein levels after IL-1β stimulation.** MCF-7 cells were transfected with GFP siRNA or p65 siRNA, and Wip1 and p65 protein levels were measured with and without 24 hours of IL-1β stimulation. The cells were harvested two, four, and six hours later. Immunoblot analysis of the cell lysates show that total Wip1 protein begins to decrease at two hours and substantially decreases by four hours after IL-1β stimulation (Figure 14). These results show that Wip1 expression is negatively regulated by IL-1β (at least at early times after IL-1β stimulation) and, since IL-1β is a strong NF-κB activator, suggest that NF-κB may be negatively regulating Wip1 expression in this context.

4.2.4 *The NF-κB p65 subunit regulates Wip1 expression after IL-1β stimulation.*

In order to test whether NF-κB has a role in modulating Wip1 expression by IL-1β stimulation, siRNA directed toward the p65 subunit was transfected into MCF-7 cells. As seen in Figure 15, knock-down of p65 expression by siRNA reduces total Wip1 protein basal levels as expected. Consistent with the above results, total Wip1 protein harvested from cells stimulated with IL-1β (Figure 15, “GFPi” under “IL-1β, 24 h”) is reduced compared to control cells (Figure 15, “-”). The decline of Wip1 expression seems to be time dependent, since Wip1 expression was more dramatically decreased
twenty-four hours after IL-1β stimulation (Figure 15) than at the early times (Figure 14). Furthermore, p65 knock-down with siRNA treatment reduced the IL-1β-dependent inhibition of Wip1 expression (Figure 15, “p65i” under “IL-1β, 24 h”). These data indicate that p65, at least in part, is responsible for the negative regulation of Wip1 expression by IL-1β.

4.2.5 Wip1 promoter activity is reduced after IL-1β stimulation.

Since IL-1β reduces Wip1 protein levels, PPM1D transcription may also be affected. In order to test this, the reporter construct described in Chapter 2, “pWip1-luc,” was utilized. The construct was transfected into HCT-116 cells, and IL-1β was added to the media twenty-four hours later. Luciferase activity, which is indicative of PPM1D promoter activity, was then measured at various times after IL-1β addition. As seen in Figure 16, PPM1D promoter activity was decreased to about 60% two and six hours after IL-1β stimulation.
in wild type cells ("wild type"), but it was more dramatically decreased at four hours (~20%). Both PPM1D promoter activity and Wip1 protein levels show the same relative decrease at early times after IL-1β stimulation, which indicates that Wip1 expression is being regulated at the level of transcription.

4.2.6 Wip1 promoter activity is regulated by p53, p65, and C/EBPα after IL-1β stimulation.

The p53 dependence of IL-1β–regulated Wip1 expression was tested next since Wip1 is known to be regulated by p53 (5,36), and p53 is activated after cytokine stimulation (113,114). For example, p53 is activated by phosphorylation and acetylation at early times by β-Interferon, which can be seen at four and six hours after stimulation (114). Whether p53 regulates PPM1D promoter activity after IL-1β stimulation was tested by using HCT-116 cells with the p53 gene deleted. PPM1D promoter activity was decreased to the same extent in both wild type and p53-/- HCT-116 cells at two and six hours after IL-1β stimulation. However, there was a more significant reduction in the PPM1D promoter activity in the wild type HCT-116 cells at four hours after IL-1β stimulation than in the p53-/- cells (Figure 17A). At four hours, wild type cells showed about an eighty percent decrease in PPM1D promoter activity, whereas PPM1D promoter activity was only reduced about forty percent in the p53-/- cells (Figure 17A). The fact that the loss of p53 partially rescues the drop in PPM1D promoter activity in response to IL-1β indicates that p53 negatively regulates PPM1D transcription four hours
Figure 17

p65, p53, and C/EBPα repress PPM1D promoter activity at different times after IL-1β stimulation. A) Luciferase activity (indicative of PPM1D promoter activity) was measured at the indicated time points after IL-1β stimulation in wild type and p53/-HCT-116 cells. Wild type and mutant constructs (described in the text) were used to measure PPM1D promoter activity B) two hours, C) four hours, or D) six hours after IL-1β stimulation in wild type and p53/- HCT-116 cells. Values are normalized to basal luciferase activity, which is set to one.
after IL-1β stimulation. Since p65 only partially inhibits the IL-1β-dependent reduction of Wip1 expression (Figure 15) and p53 seems to only regulate PPMID promoter activity at certain times after stimulation (Figure 17A), multiple transcription factors are likely responsible for IL-1β-regulated PPMID promoter activity.

To further analyze the regulation of PPMID promoter activity after IL-1β stimulation, additional PPMID promoter constructs were utilized. As described in Chapter 3, a κB site is located at -793 base pair position. Additionally, there is an evolutionarily conserved binding site near the κB site for the transcription factor C/EBPα at -806 base pair position (Figure 18). The fact that the κB and the C/EBPα sites are in close proximity to each other in the promoter is interesting, especially since NF-κB and C/EBP coordinate to modulate the transcription of certain NF-κB target genes such as IL-6 and IL-8 (106,108). In order to test whether p65 and C/EBPα, like p53, regulate the transcription of PPMID after IL-1β, luciferase reporter constructs with the deletion of either the κB site, the C/EBPα site, or both were utilized (Figure 18). The constructs were transfected into wild type and p53/-/ HCT-116 cells, and IL-1β was added to the media twenty-four hours later. Luciferase was measured at two, four, and six hours after IL-1β addition. As seen in Figure 17B, the deletion of either the κB site, the C/EBPα site, or both had no effect on the IL-1β-regulated PPMID promoter activity in both wild type and p53/-/ cells two hours after stimulation. These results demonstrate that other transcription factors are responsible for the decrease in PPMID promoter activity at two hours after stimulation and not p53, C/EBPα, or NF-κB.
At four hours after IL-1β stimulation, deletion of the κB, the C/EBPα site, and both completely removes the inhibition of PPM1D promoter activity in p53-/− cells.

<table>
<thead>
<tr>
<th>Name</th>
<th>κB location</th>
<th>Predicted C/EBPα site (location, distance from κB site)</th>
</tr>
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<tbody>
<tr>
<td>Human (Homo sapiens)</td>
<td>-793 bp</td>
<td>C/EBPα (-806 bp, 13bp)</td>
</tr>
<tr>
<td>Chimpanzee (Pan troglodytes)</td>
<td>-791 bp</td>
<td>C/EBPα (-804 bp, 13bp)</td>
</tr>
<tr>
<td>Mouse (Mus musculus)</td>
<td>-798 bp</td>
<td>C/EBPγ (-781 bp, 17bp)</td>
</tr>
<tr>
<td>Zebrafish (Danio rerio)</td>
<td>-970 bp</td>
<td>C/EBPα (-961 bp, 9bp)</td>
</tr>
</tbody>
</table>

**Figure 18**

**Location of the κB and C/EBP binding site in the PPM1D promoter region and reporter construct design.** A) The location of the κB and C/EBP binding site is evolutionarily conserved. B) Schematic of the PPM1D reporter construct design.

However, there is no effect of the deletion of any of the sites on PPM1D promoter activity in wild type cells (Figure 17C). The fact that deletion of the κB or the C/EBPα sites alone rescues the inhibition of promoter activity in p53-/− cells indicates that NF-κB and C/EBPα cooperate to repress PPM1D transcription independently of p53. Additionally, p53 regulation of PPM1D promoter activity may be strong enough to
compensate for the loss of the regulation by NF-κB and C/EBPα, which may be the reason why there is no effect seen with the deletion of both sites in the wild type cells at four hours post-stimulation.

At six hours after IL-1β stimulation, deletion of the κB and the C/EBPα sites rescued *PPM1D* promoter inhibition (Figure 17D), which indicates that NF-κB and C/EBPα repress *PPM1D* promoter activity beginning at four hours after stimulation. On the other hand, *PPM1D* promoter activity is not altered by deleting p53 at six hours after stimulation, indicating that other unidentified transcription factors regulate promoter activity along with NF-κB and C/EBPα at this time point.

### 4.3 Discussion

The results in this section show that regulation of Wip1 expression after various NF-κB stimuli is complex and depends on the type and length of stimulation. The results are in direct contrast to those presented in Chapter 3 and show that in some cases NF-κB negatively regulates Wip1 expression.

The fact that the inverse correlation between Wip1 expression and p38 activation is maintained despite the differences in Wip1 expression at early versus late time points after LPS stimulation in splenic lymphocytes is intriguing. The results in Chapter 2 explain, in part, the functional reason for a decrease in p38 activity and increase in Wip1 expression at late times after LPS stimulation. Inactivation of p38 at late times after stimulation is necessary for full proliferation, and higher levels of Wip1 probably ensure that this happens. On the other hand, the role of active p38 at early time points is to
facilitate B-cell signaling, for example by regulating cytokine expression (87,88).

However, the reason for the marked reduction of Wip1 expression at early times after LPS stimulation is unknown. One reason for Wip1 expression inhibition could be to ensure that p38 activity is not turned-off prematurely at the early time points since p38 is a Wip1 target, although this is purely speculative and requires empirical validation.

NF-κB was shown to increase Wip1 expression at late times after LPS stimulation; however, the mechanism of Wip1 inhibition at early times is unknown. LPS/TLR4 signaling has two major pathways, MyD88-dependent early signaling and MyD88-independent late signaling. Both lead to the activation of NF-κB, but the early MyD88-dependent signaling also activates p38 and c-jun (112). It is possible that the early inhibition of Wip1 expression after LPS stimulation is due to p38 or c-jun, and not NF-κB. Another possibility is that certain early phase and late phase co-factors cooperate with the NF-κB subunits to differentially regulate PPM1D transcription.

The IL-1β studies illustrate the complexity of PPM1D transcription after cytokine stimulation. Three different transcription factors (p53, p65, and C/EBPα) inhibit Wip1 expression, and do so at different times after stimulation. p65 seems to cooperate with C/EBPα to attenuate PPM1D transcription at four and six hours after IL-1β stimulation. p53 inhibits transcription at four hours after stimulation, and this regulation seems to be strong enough to compensate for the loss of p65 and C/EBPα regulation. On the other hand, none of these factors regulate PPM1D transcription two hours after IL-1β stimulation. Since multiple transcription factors are responsible for the regulation of
Wip1 expression after IL-1β stimulation, different stimuli (especially cytokine stimulation) probably have distinct signature effects on Wip1 expression.

The fact that p53 appears to regulate PPM1D transcription most prominently at four hours after stimulation is interesting and consistent with previously published results on the timing of p53 activation after cytokine stimulation and exposure to genotoxic stress. Although, the current literature does not describe a time course of p53 activation after IL-1β stimulation, stimulation with β-IFN induces p53 activation in two waves that is similar to LPS-induced NF-κB activation (114). The first wave of p53 activation occurs at early times (around four to six hours after stimulation) and involves p53 acetylation. The second wave leads to p53 activation in the same manner as genotoxic stress (114). Additionally, PPM1D transcription is regulated by p53 most prominently at four hours after IR exposure (36). The times of activation of p53 in these contexts is consistent with the time that the IL-1β-induced PPM1D promoter activity regulation becomes p53-dependent. Although, it is necessary for future studies to establish whether this pattern of p53 activation also exists in IL-1β signaling, since the signaling of cytokines is different.

In summary, these results show that regulation of PPM1D transcription by NF-κB does not always lead to an increase in Wip1 expression. Whether NF-κB positively or negatively regulates Wip1 expression depends on the cellular context including the type of stimulus. Furthermore, the regulation of Wip1 expression after different cytokine stimulation likely is not limited to regulation by NF-κB, which is indicated by the results
with IL-1β stimulation and the fact that different cytokines activate multiple transcription factors. These factors, therefore, should be taken into account when analyzing the regulation of Wip1 expression after different types of stimulation.
Chapter 5. Wip1 inhibits Non-Homologous End-Joining (NHEJ) by inactivating DNA-PK.

5.1 Introduction

IR exposure induces multiple types of DNA lesions, and the most lethal type of lesion is the double strand break (DSB). Cellular death results if DSBs are not repaired, and unsuccessful repair of DSBs can lead to gross chromosomal aberrations, which may lead to genomic instability, the expression of non-functional proteins, and possibly tumorigenesis (115). There are two types of DSB repair, Homologous Recombination and Non-Homologous End-joining (NHEJ), and NHEJ is the predominant type of DSB repair (35). NHEJ is a process that utilizes multiple repair factors including the DNA-dependent-protein kinase (DNA-PK) complex of proteins. Briefly, DNA-PK assembles at the DSB – the Ku 70 and Ku80 regulatory subunits bind to the free ends of the break, and then the catalytic subunit (DNA-PKcs) is recruited. A synapsis is formed by the DNA-PK complexes on both ends of the break, and active DNA-PK facilitates end processing by recruiting and/or activating multiple proteins including Artemis and Werner. Next, XRCC4 and DNA Ligase IV are recruited to seal the gap and restore the double stranded DNA (116,117).

DNA-PK is a member of the PI3K-like kinases and is crucial to the NHEJ process, since cells that do not express DNA-PK exhibit a radiosensitive phenotype and have deficient DSB repair (116). DNA-PK function is regulated by the binding of the Ku regulatory subunits to DNA-PKcs and by phosphorylation of DNA-PKcs. DNA-PKcs is a large protein (~460 kDa) and is phosphorylated on multiple residues (117). There are
two main clusters of phosphorylation sites – one is between residues 2609 and 2647 (the “ABCDE cluster”) and the other is between residues 2023 and 2056 (the “PQR cluster”) (4,118-120). DNA-PKcs phosphorylation is complex and not well-understood since there are multiple phosphorylation sites on the protein (besides these two clusters) and there are unknown sites that are important for full DNA-PKcs function (117,121). However, the current literature indicates that the ABCDE cluster phosphorylation is important in end processing and crucial to NHEJ (117). Furthermore, phosphorylation of threonine 2609 (site “A,” visualized with immunofluorescence) was seen to colocalize at damage sites with γ-H2AX and has been used as an indicator of active DNA-PK (122).

The results in this chapter show that Wip1 reduced phosphorylation of DNA-PKcs on an activating residue (Thr2609), which was assessed by immunofluorescence and immunoblot analysis. Finally, Wip1 inhibits NHEJ, presumably through its effects on DNA-PK.

5.2 Results

5.2.1 DNA-PKcs has conserved Wip1 binding sites

One of the consensus binding sequences for Wip1 is a SQ/TQ motif (the serine or threonine is the residue that is phosphorylated and a glutamine is in the +1 position) preceded by acidic residues (2,68). This motif is characteristic of a Wip1 binding site since the glutamine in the +1 position and the N-terminal acidic residue appears necessary for proficient Wip1 binding (68). Since DNA-PKcs also has SQ/TQ motifs, sequences surrounding known phosphorylation sites of DNA-PKcs were analyzed for Wip1 consensus binding sequences. Figure 19 shows the DNA-PKcs conserved ABCDE
phosphorylation sites. Site “A,” the T2609 residue, is a TQ motif with a glutamic acid (E) in the region upstream of the site (N-terminal region) that is also evolutionarily conserved (Figure 19, boxed in red). Since this binding site shares characteristics of the Wip1 consensus binding sites (SQ/TQ motif), the T2609 phosphorylation site of DNA-PKcs is possibly a target of Wip1.

5.2.2 Wip1 reduces phosphorylation of DNA-PKcs at T2609.

Since an antibody against phosphorylated DNA-PKcs at Thr2609 is commercially available and suitable to visualize IR-induced phospho-DNA-PKcs foci, immunofluorescence was performed using this antibody to determine if Wip1 reduces phosphorylation of this site. For these experiments, H1299 cells genetically altered to express Wip1 in response to doxycycline treatment were used. H1299 cells were

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Figure 19

The T2609 site in the ABCDE phosphorylation cluster region is a candidate Wip1 binding site. The ABCDE phosphorylation sites are evolutionarily conserved. The “A” site, T2609, has a conserved TQ motif and a conserved acidic glutamate (“E”) residue N-terminal from the T residue, which are characteristics of Wip1 binding sites (highlighted by a red rectangular box). Adapted from (4).
Figure 20

**Wip1 reduces IR-induced phospho-DNA-PKcs (T2609) foci.** H1299 cells expressing Wip1 (white arrows) show a reduction in foci corresponding to phospho-DNA-PKcs (T2609) (“pDNA-PKcs”) at the indicated time points after IR exposure. DAPI is used as a nuclear stain. (Wip1-green, pDNA-PKcs-red, DAPI-blue)
incubated with doxycycline to express Wip1 and then either sham irradiated or exposed to IR sixteen hours later. The cells were harvested at various times after exposure and prepared for immunofluorescence using the phospho-DNA-PKcs (T2609) antibody. A minority of the tet-on Wip1 inducible H1299 cells fail to express Wip1 after doxycycline treatment. Since these cells can serve as internal negative controls, select fields including these infrequent doxycycline-unresponsive cells were chosen. As seen in Figure 20, IR exposed cells that do not express Wip1 (Figure 20, white arrowheads) show phospho-DNA-PKcs foci at one hour and persisting until four hours after IR. However, these foci are reduced in cells expressing Wip1 (Figure 20, white arrows), at one, two, and four hours after IR.

To ensure the specificity of this antibody, immunofluorescence was performed with these cells with or without incubation of a phospho-DNA-PKcs (T2609) blocking peptide. As seen in Figure 21, no basal pDNA-PKcs (T2609) foci are seen in all cells regardless of Wip1 expression (“0 Gy” panels). As expected, cells not expressing Wip1 show pDNA-PKcs (Thr2609) foci after IR exposure, which were completely removed with incubation of the blocking peptide (Figure 21). Consistent with the results in Figure 20, Wip1 expressing cells show a reduction in pDNA-PKcs (T2609) foci two hours after IR (Figure 21). These results confirm that the phospho-DNA-PKcs (Thr2609) antibody is specific and show that Wip1 reduces IR-induced phosphorylation of DNA-PKcs at Thr2609.
**Figure 21**

**Anti-pDNA-PKcs (T2609) antibody is specific.** H1299 cells with and without Wip1 induction ("+" or "-" Dox, respectively) were either sham irradiated ("0 Gy") or harvested 2 hours after IR exposure ("5 Gy, 2 hr"). Immunofluorescence was
Figure 21 (cont.) performed with the pDNA-PKcs (T2609) antibody (“pDNA-PK”) with or without treatment with the p-DNA-PKcs (T2609) blocking peptide (“+” or “-” blocking peptide, respectively) to test the antibody specificity. (Wip1-green; pDNA-PK-red; DAPI-blue)

In order to complement the immunofluorescence results above, immunoblot analysis was performed. Lysates from H1299 cells treated with doxycycline to induce Wip1 expression were probed for p-DNA-PKcs (T2609) with and without IR exposure by immunoblot analysis. Figure 22 shows that the level of phosphorylated DNA-PKcs on Thr2609 after IR increases as expected in the control cells (“- dox”). However, IR-induced p-DNA-PKcs (T2609) levels are reduced in H1299 cells expressing Wip1 (Figure 22, “+ Dox”). Taken together with the immunofluorescence images, these results show that Wip1 reduces IR-induced phosphorylation of DNA-PKcs on T2609.

<table>
<thead>
<tr>
<th>pT2609 DNA-PKcs</th>
<th>0 Gy</th>
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<tr>
<td>Dox</td>
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<tr>
<td>DNA-PKcs</td>
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<td>Ku86</td>
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Wip1 reduces IR-induced phospho-DNA-PKcs (T2609). Levels of phospho-DNA-PKcs T2609, total DNA-PKcs, and Ku86 (loading control) protein in H1299 cells expressing Wip1 (“+ Dox”) or not (“- Dox”) and unexposed (“0 Gy”) or harvested two hours after IR (“5 Gy”) were analyzed by immunoblot analysis.
To ensure that the effect of Wip1 on phosphorylated DNA-PKcs is not limited to H1299 cells, similar experiments were performed using BJhTert fibroblasts and HCT-116 colon carcinoma cells. These cells were transiently transfected with a Flag-tagged Wip1 expression vector and exposed to IR twenty-four hours later. At two hours after exposure, phospho-DNA-PKcs (Thr2609) foci were visualized (Figure 23). Like in the H1299 cells, expression of Wip1 resulted in a reduction of IR-induced phospho-DNA-PKcs (T2609) foci in BJhTert cells and HCT116 cells (Figure 23).
Figure 24

Wip1 reduction of p-DNA-PKcs (T2609) requires Wip1 phosphatase activity.
5.2.3 **Wip1 phosphatase activity is required for reduction of IR-induced p-DNA-PKcs (T2609) foci.**

If Wip1 reduces phospho-DNA-PKcs (T2609) by direct dephosphorylation, then Wip1 phosphatase activity should be required. To test this, stable H1299 cell lines made with either an empty vector, a Wip1 expression vector, or a mutated Wip1 expression vector (D314A) that lacks phosphatase activity were used. These cells were exposed to IR and prepared for immunofluorescence one and two hours later. As seen in Figure 24, cells over-expressing Wip1 showed reduced p-DNA-PKcs (T2609) foci at one and two hours after IR. However, over-expression of the phosphatase dead mutant Wip1 (Figure 24, “Wip1DA”) did not result in a decrease in p-DNA-PKcs (T2609) foci. These results indicate that Wip1 phosphatase activity is required to decrease IR-induced DNA-PKcs phosphorylation and suggest that Wip1 dephosphorylates DNA-PKcs.

5.2.4 **Wip1 reduces NHEJ**

The fact that Wip1 reduces an activating DNA-PKcs phosphorylation that is required for full DNA-PK function suggests that Wip1 reduces DNA-PK function. Since DNA-PK is essential for NHEJ, Wip1 may have an inhibitory effect on NHEJ. In order
to test this, tet-on Wip1 inducible H1299 cells were used. Extracts were harvested from cells induced to express Wip1 (“+ dox”) or not (“- dox”) and either sham irradiated (“sham”) or exposed to IR (harvested thirty minutes after exposure, “5 Gy, 30 min”). These extracts were used in an in vitro end-joining assay following a previously described protocol (123). Briefly, extracts are incubated with linearized plasmid DNA, and then the amount of linear DNA after incubation with the extracts is visualized after running the samples on an agarose gel. Therefore, an increase in end-joining ability of extracts would be seen as a decrease in the amount of linear plasmid DNA (and higher amount of mended high molecular weight DNA). As seen in Figure 25, extracts from the sham irradiated Wip1-expressing cells (“+dox, sham”) have a decreased ability to mend DSBs in vitro compared to the control (“-Dox, sham”) as seen by an increase in linear DNA. Additionally, expression of Wip1 reduces the levels of in vitro end-joining after IR (“+dox, 5 Gy, 30 min”) compared to the non-Wip1 expressing irradiated controls (“-dox, 5 Gy, 30 min,”) since there are higher levels of linear plasmid

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**Figure 25**

**Wip1 reduces NHEJ basally and after IR exposure.** H1299 cells expressing Wip1 (“+Dox”) or not (“-Dox”) were either sham irradiated (“sham”) or harvested thirty minutes after IR (“5 Gy, 30 min”). Extracts were used in in vitro end-joining assays. (“Input DNA,” DNA without cell extracts) *Figure generated by Silvia Pandolfi, Ph.D, unpublished data.
DNA in end-joining reactions using the Wip1 expressing cell lysates (Figure 25). These results indicate that Wip1 reduces the ability of a cell to mend DSBs by NHEJ both basally and after IR exposure, presumably by dephosphorylating and inhibiting DNA-PK function.

5.3 Discussion

The results in this chapter show that Wip1 targets DNA-PK, particularly DNA-PKcs at the phosphorylated T2609 site (“A” site), and as a consequence inhibits NHEJ. Wip1 may directly dephosphorylate DNA-PKcs on the T2609 residue for the following reasons. The sequence surrounding the T2609 site is consistent with the Wip1 consensus sequence, which suggests that Wip1 may be able to bind to the T2609 site. Wip1 inhibition of an upstream kinase can be excluded, since phosphorylation of the T2609 residue has been shown to occur by autophosphorylation (124). Additionally, this site is apparently easily accessible since it is targeted by other phosphatases such as PP2A, PP5, and PP6 (125-127). On the other hand, it is possible that Wip1 may reduce T2609 phosphorylation by directly dephosphorylating other residues of the ABCDE cluster since some sites (such as “D” and “E,” Figure 19) are also SQ/TQ motifs (albeit without the N-terminal acidic residues). It has been suggested that phosphorylation of one site may lead to phosphorylation of other sites within the cluster (117), and dephosphorylation of another site of the ABCDE cluster by Wip1 may lead to a reduction of T2609 phosphorylation (117). Therefore, while direct targeting of Wip1 to the T2609 site needs to be validated, the results presented in this chapter indicate that Wip1 presumably
inhibits DNA-PKcs end processing ability by regulating ABCDE cluster phosphorylation as seen by a reduction of T2609 phosphorylation.

Regulation of DNA-PKcs function is most likely mediated through multiple phosphorylation sites (not just one site alone), since dephosphorylation of DNA-PKcs by PP2A and Wip1 result in either the activation or the inhibition of DNA-PKcs activity, respectively (125). The fact that PP2A activates DNA-PKcs by dephosphorylation (125) is in contrast to the results presented here, since dephosphorylation of DNA-PKcs by Wip1 presumably inhibits DNA-PK activity. The discrepancy between the effects of Wip1 and PP2A on DNA-PKcs activity is probably attributed to the fact that each phosphatase affects a number of phosphorylation sites (and not just T2609). It has already been shown that phosphorylation of all of the sites within a cluster (not only one site) seems to be important for DNA-PKcs function, since mutation of all of the sites together (but not the mutation of each site alone) affects DNA-PKcs function and DSB repair (117). Additionally, there are currently unknown phosphorylation sites on the DNA-PKcs protein that are functionally important (117,128). Therefore, inactivation of DNA-PKcs by Wip1 is probably a result of dephosphorylation of multiple sites, and the fact that both Wip1 and PP2A reduce DNA-PKcs phosphorylation but differentially regulate its activity illustrates the complexity of DNA-PKcs phosphorylation.

The opposing effect of Wip1 and PP2A on DNA-PKcs is not surprising since Wip1 is oncogenic and PP2A has tumor suppressor properties. Dephosphorylation of DNA-PKcs by PP2A has been shown to activate DNA-PKcs, which likely promotes
genetic stability by inhibiting gross chromosomal aberrations that result from NHEJ inhibition after genotoxic stress (125). On the other hand, dephosphorylation of DNA-PKcs by Wip1 leads to an inhibition of NHEJ. Wip1 inhibition of NHEJ may promote genomic instability, especially in cells that have Wip1 amplified and over-expressed. Therefore, an additional mechanism by which Wip1 promotes tumorigenesis may be to enhance genomic instability by dephosphorylating and inactivating DNA-PKcs.

Finally, inhibition of DNA-PKcs by Wip1 may be important once DSBs are repaired and NHEJ has to be shut down. Wip1 inactivates several stress signaling pathways to inhibit apoptosis and reverse cell cycle arrest (6). Given that these effects of Wip1 are prominent after genotoxic stress, it is surprising that there is currently only a single report on the effect of Wip1 on DNA repair showing that Wip1 inhibits BER by dephosphorylating a uracil glycosylase (25). The studies described here illustrates that Wip1 inhibits NHEJ, which not only may enhance genomic instability when Wip1 is over-expressed but also may be necessary to return a stressed cell to homeostasis once damage is repaired.
Chapter 6. Wip1 positively regulates NF-κB signaling.

6.1 Introduction

The above chapters describe evidence that NF-κB regulates Wip1 expression. Additionally, Chew et al, show that Wip1 negatively regulates NF-κB signaling and, therefore, inhibits inflammatory signaling to facilitate the return of the cell to homeostasis after immunological challenges (46). However, as described in Chapter 4, NF-κB signaling is complex, and there is evidence in the literature that Wip1 may positively regulate NF-κB signaling. The transgenic Wip1-/- mouse exhibits distinct phenotype characteristics (27) that have not yet been fully explained in the literature, and Wip1 complementation of the NF-κB pathway may be the reason for these phenotypes. For example, (1) Wip1-/- mouse embryonic fibroblasts (MEFs) exhibit growth defects in culture, (2) Wip1-/- mice are more susceptible to infection, and (3) Wip1-/- mouse splenic lymphocytes have decreased proliferation in response to lipopolysaccharide (LPS) and phytohemoglutin (PHA) compared to wild type (27). Atypical activity of the NF-κB transcription factors may account for, at least in part, the attributes described since this pathway is known to play an important role in cellular growth, in immune system function, and in T- and B-cell proliferation in response to stimuli (67,76,86,129). Additionally, NF-κB activation and Wip1 induction coincide in time after genotoxic stress (such as IR (5,130)). Augmentation of NF-κB signaling by Wip1 after genotoxic stress makes sense since it would result in increased survival cues. Therefore, Wip1 may enhance NF-κB signaling in some contexts.
In this chapter, results are shown that demonstrate Wip1 as a positive regulator of NF-κB signaling by using cell lines in which Wip1 is either deleted or over-expressed and reporter constructs to measure NF-κB activity. Additionally, expression of Wip1 leads to a reduction of expression levels of the NF-κB inhibitor, IκBα, at the protein and mRNA levels. Likewise, deletion of Wip1 enhances IκBα protein and mRNA levels. Finally, deletion of Wip1 enhances IκBα mRNA stability. These data show that in some contexts Wip1 enhances NF-κB activity by reducing IκBα expression.

6.2 Results

6.2.1 Overexpression of Wip1 enhances basal NF-κB activity

Constitutive activation of NF-κB has been shown to be important in the survival and growth of several human cancers including prostate cancer (131-135). Since Wip1 is over-expressed in several cancers including prostate cancer (6,12), the effect of Wip1 expression on NF-κB activity in the prostate cancer cell lines DU-145 and PC-3 was evaluated. A reporter construct including κB sites upstream of a luciferase gene was used to measure NF-κB activity, and this measurement was normalized to the activity of translation elongation factor 1 alpha (EF1a), which is a housekeeping gene that facilitates protein translation (136). DU-145 and PC-3 cells were co-transfected with a Wip1 expression vector and either the NF-κB reporter construct or the EF1a reporter construct,
and luciferase activity was measured twenty-four hours later. As seen in Figure 26, over-expression of Wip1 in both DU-145 cells (Figure 26A) and PC-3 cells (Figure 26B) resulted in a significant increase in basal NF-κB activity. These results show a direct correlation of Wip1 expression and NF-κB activation and indicate that Wip1 enhances constitutive basal NF-κB activity.

6.2.2 Deletion of Wip1 reduces basal NF-κB activity.

Since over-expression of Wip1 enhanced NF-κB activity, loss of Wip1 expression should decrease NF-κB activity. In order to test this, mouse embryonic fibroblasts that were transformed with the oncogenes E1A and H-Ras1 and harvested from either wild
Deletion of Wip1 reduces NF-κB activity. Wild type and Wip1-/- E1A/Ras MEFs were transfected with a NF-κB (or EF1a) luciferase reporter construct, and luciferase activity was measured twenty-four hours later.

**Figure 27**

Deletion of Wip1 reduces NF-κB activity. Wild type and Wip1-/- E1A/Ras MEFs were transfected with a NF-κB (or EF1a) luciferase reporter construct, and luciferase activity was measured twenty-four hours later.

*Figure 27*

**Deletion of Wip1 reduces NF-κB activity.** Wild type and Wip1-/- E1A/Ras MEFs were transfected with a NF-κB (or EF1a) luciferase reporter construct, and luciferase activity was measured twenty-four hours later.

Taken together with the over-expression studies in the section above, these results show that Wip1 positively regulates NF-κB activity in cells undergoing oncogenic stress, which suggests that Wip1 regulation of NF-κB may be an additional oncogenic function of Wip1.

6.2.3 Wip1 enhances IR-induced NF-κB activation.

Not only does NF-κB play a role in basal tumor cell proliferation, but enhanced NF-κB activity also increases the radioresistance of cancer cells since NF-κB is activated after IR exposure (69-72,95-97,99,101,137,138). Therefore, the effect of Wip1 on IR-induced NF-κB activity was next measured by using the E1A/Ras MEFs and tet-on Wip1 inducible H1299 cells, which are genetically altered to induce Wip1 expression in the presence of doxycycline. To determine whether deletion of Wip1 affected IR-induced NF-κB activity, the NF-κB reporter construct (or the EF1a reporter construct as a control)
Wip1 enhances IR-induced NF-κB activity. NF-κB activity (measured as luciferase activity and normalized to EF1a) was measured at the indicated time after IR exposure in A) wild type and Wip1-/- E1A/Ras MEFs, B) tet-on Wip1 inducible H1299 cells that express Wip1, and C) HCT-116 cells over-expressing Wip1 ("Wip") or not ("Flag"). In B), the y-axis is the fold change of NF-κB activity in Wip1 expressing cells compared to non-Wip1 expressing cells (+dox/-dox).

was transfected into both wild type E1A/Ras MEFs and Wip1-/- E1A/Ras MEFs. These cells were exposed to IR twenty-four hours after transfection, and luciferase activity was
measured at the indicated times after IR exposure. As expected, NF-κB was activated at early times (two and four hours) after IR exposure in wild type E1A/Ras MEFs as seen by an increase in luciferase activity (Figure 28). On the other hand, IR-induced NF-κB activation was absent in Wip1-/ E1A/Ras MEFs since luciferase activity was the same as basal at all times after IR exposure (Figure 28). These results show that deletion of Wip1 abolished IR-induced NF-κB activity, suggesting that Wip1 is an important regulator of NF-κB activation after IR.

Next, the effect of Wip1 over-expression on IR-induced NF-κB activation was tested in the tet-on Wip1 inducible H1299 cells. H1299 cells were transfected with the NF-κB reporter constructs and doxycycline was added to the media to stimulate Wip1 expression. Luciferase activity in the cells was measured at one, two, and four hours after IR exposure. As seen in Figure 28, Wip1 expression lead to an increase in IR-induced activity at all times measured compared to the non-Wip1 expressing cells. IR-induced NF-κB activation increased two-fold at one hour post-IR, and by four hours the increase was greater than four-fold (Figure 28). A similar effect was seen in the colon carcinoma HCT-116 cells – cells transfected with a Wip1 expression vector had a significant increase in IR-induced NF-κB activity at one, two, four, and twenty-four hours after IR exposure compared to control cells (Figure 28). These results show that Wip1 enhances IR-induced NF-κB activation.
6.2.4 Wip1 enhances TNFα-induced NF-κB activation

As explained in previous chapters, TNFα is a well-characterized NF-κB activator (82,83). Since Wip1 enhances NF-κB activity basally and after IR exposure, the effect of Wip1 on TNFα-induced NF-κB activation was tested in both DU-145 and PC-3 cells. Wip1 over-expression enhanced basal NF-κB activation as expected in both cell types (Figure 29). In DU-145 cells, Wip1 expression significantly increased TNFα-induced NF-κB activation one and two hours after stimulation, and basal levels of NF-κB activity were restored in both Wip1-expressing (Figure 29B, “Wip1”) and non-Wip1 expressing
Figure 30

**Deletion of Wip1 enhances IκBα expression.** A) and B) IκBα mRNA levels were measured by qRT-PCR and C) IκBα protein was measured by immunoblot analysis in wild type and Wip1-/- E1A/Ras MEFs without IR (“-”) and at the indicated time after IR exposure. B) Wild type IκBα mRNA levels are graphed with a smaller y-axis scale. IκBα mRNA values are normalized to GAPDH, and β-actin is used as a loading control.

Therefore, Wip1 over-expression augments TNFα-induced NF-κB activation.
6.2.5 **Deletion of Wip1 enhances IκBα expression.**

The results above establish Wip1 as a positive modulator of NF-κB. In order to find the mechanism by which Wip1 regulates NF-κB, the effect of Wip1 on IκBα expression levels was tested. IκBα inhibits NF-κB by preventing nuclear translocation (67), and Wip1 may enhance NF-κB activity by decreasing IκBα expression. Therefore, IκBα expression was measured in wild type and Wip1-/- E1A/Ras MEFs with and without IR exposure. Basal IκBα expression was dramatically higher in Wip1-/- E1A/Ras MEFs compared to wild type E1A/Ras MEFs (Figure 30A, “-”), indicating that Wip1 negatively regulates IκBα mRNA levels. This difference in basal IκBα mRNA levels, however, is quite dramatic, and future validation of these results by northern blot analysis is necessary. Additionally, the expected IκBα mRNA pattern after IR exposure is seen in wild type E1A/Ras MEFs (Figure 30B), which mimics that of IR-induced NF-κB activation. Shortly after IR exposure, activated NF-κB enhances IκBα transcription, and then the rise in IκBα levels returns NF-κB activity to basal levels, which then decreases IκBα to basal levels. On the other hand, this IκBα mRNA pattern is lost in the Wip1-/- E1A/Ras MEFs, and IκBα mRNA levels appear to even decrease initially after IR exposure (Figure 30A).

Next, basal IκBα protein and IκBα protein levels after IR exposure were evaluated in wild type verses Wip1-/- E1A/Ras MEFs. Like IκBα mRNA, the expected pattern of IκBα protein levels is seen in wild type E1A/Ras MEFs (Figure 30C). After IR exposure, NF-κB is activated after IκBα protein is degraded, which frees the NF-κB
Over-expression of Wip1 decreases IκBα expression. A) IκBα mRNA levels were measured by qRT-PCR and B) IκBα protein levels were measured by immunoblot analysis in PC-3 cells that were transfected with the Flag empty vector (“Flag”) or the Flag-tagged Wip1 expression vector (“Wip1”). In B), IκBα protein was also measured in lysates from PC-3 cells harvested at the indicated time after IR exposure. IκBα mRNA values were normalized to GAPDH, and β-actin is used as a loading control.

Figure 31

subunits to translocate into the nucleus. Active NF-κB then increases IκBα transcription and, therefore, protein. Therefore, IκBα protein decreases at early times after IR exposure due to degradation and then increases at later time points once NF-κB is activated and enhances IκBα transcription (Figure 30C). However, basal IκBα protein levels in Wip1−/− E1A/Ras MEFs are much higher than in the wild type cells, and the levels remain high after IR exposure (Figure 30C). The fact that the pattern of IκBα expression after IR is disrupted in the Wip1−/− E1A/Ras MEFs is additional evidence that deletion of Wip1 disrupted IR-induced NF-κB activation. Additionally, the dramatic increase in
IκBα expression levels both basally and at all times after IR exposure indicates that Wip1 may be affecting NF-κB activity by modulating IκBα expression.

6.2.6 Over-expression of Wip1 decreases IκBα expression.

Since deletion of Wip1 increased IκBα expression, over-expression of Wip1 should decrease IκBα expression. In order to test this, a Wip1 expression vector was transiently transfected in PC-3 cells, and IκBα mRNA levels were measured twenty-four hours later. Cells that over-express Wip1 (“Wip1,” Figure 31A) showed a decrease in IκBα mRNA levels compared to cells transfected with an empty vector (“Flag,” Figure 31A). Additionally, evaluation of IκBα protein levels in these cells revealed a decrease in Wip1 over-expressing cells, especially after IR exposure (Figure 31B). The fact that IκBα protein decreases after IR in cells over-expressing Wip1 is counterintuitive, since IκBα protein levels would be expected to increase after IR due to an increase in NF-κB activity by high levels of Wip1. However, ectopic expression of Wip1 by transient transfection of an expression vector yields high levels of Wip1, and endogenous Wip1 is expected to be induced after IR exposure (5). Due to these high Wip1 levels (especially after IR), Wip1 inhibition of IκBα expression may be stronger than NF-κB upregulation of IκBα transcription.

6.2.7 Deletion of Wip1 stabilizes IκBα mRNA

The results above show that Wip1 modulates IκBα expression at the mRNA level, suggesting that Wip1 could be regulating either IκBα mRNA stability or transcription of
Deletion of Wip1 enhances IκBα mRNA stability. Actinomycin D was added to the media of wild type ("wild type") and Wip1-/- ("Wip1-/-") E1A/Ras MEFs, and IκBα mRNA levels were measured at the indicated time after actinomycin D addition. IκBα mRNA levels were normalized to GAPDH, and these values were then normalized to basal IκBα mRNA levels (which are set to 1).

the IκBα gene. In order to test the ability of Wip1 to modulate IκBα mRNA stability, wild type and Wip1-/- E1A/Ras MEFs were used. The cells were incubated with actinomycin D to inhibit transcription, and mRNA of the cells was harvested at various times after actinomycin D addition. As seen in Figure 32, there is no significant difference in IκBα mRNA levels thirty minutes after actinomycin D addition between wild type and Wip1-/- E1A/Ras MEFs. However, Wip1-/- E1A/Ras MEFs had higher
levels of IκBα mRNA one, two, and four hours after actinomycin D addition than wild
type E1A/Ras MEFs (Figure 32). These results indicate that IκBα mRNA is more stable
in Wip1-/- E1A/Ras MEFs than in the wild type control cells, which explains, at least in
part, the higher basal IκBα mRNA levels seen in the Wip1-/-
E1A/Ras MEFs.

6.2.8 Wip1 increases IκBα
promoter activity.

Although deletion of Wip1
enhances IκBα mRNA stability, it
is possible that IκBα transcription
may also be affected by Wip1. In
order to test this, a reporter
construct with the IκBα promoter
region upstream from a luciferase
gene was transiently transfected
into wild type and Wip1-/-
E1A/Ras MEFs. Since IκBα mRNA levels are much higher in Wip1-/- E1A/Ras MEFs
compared to the wild type cells, IκBα promoter activity in Wip1-/- E1A/Ras MEFs
would be expected to either by higher or the same as the wild type cells. However, there
was a significant decrease in IκBα promoter activity in Wip1-/- E1A/Ras MEFs

Figure 33

Deletion of Wip1 reduces IκBα promoter
activity. The IκBα luciferase reporter construct
and a Renilla construct were co-transfected in wild
type (“wild type”) and Wip1-/- (“Wip1-/-”) E1A/Ras MEFs, and luciferase activity was
measured twenty-four hours later. IκBα promoter
activity was normalized to Renilla activity.

100
compared to the wild type cells (Figure 33). These results would be consistent with deletion of Wip1 reducing NF-κB activity if measurement of IκBα promoter activity is indicative of NF-κB activity, since IκBα is a NF-κB target. Indeed, this method of monitoring NF-κB activity is described in the literature (82,139).

Additionally, similar experiments were performed with the tet-on Wip1 inducible H1299 cells. The benefits of using these cells is that the levels of Wip1 expression can be controlled by the dose of doxycycline added to the media and the kinetics of Wip1 activation of NF-κB can be determined by monitoring NF-κB activity at various times after doxycycline addition. A time course of NF-κB-driven luciferase activity and IκBα promoter activity was performed after doxycycline addition. The cells were first transfected with the reporter constructs and doxycycline was then added to the media twenty-four hours later to induce Wip1 expression. NF-κB and IκBα promoter activity in the cells was measured at five, seven, and twenty-four hours after doxycycline addition. There was a dramatic increase in both NF-κB-driven luciferase activity (Figure 34A) and IκBα promoter activity (Figure 34B) at five hours after doxycycline addition, which then both decreased at later time points. The fact that there is a decrease in NF-κB activity at later time points compared to the five hour time point is expected since NF-κB enhances the expression of its own inhibitor, IκBα. Moreover, the fact that there is a rise
Wip1 overexpression increases NF-κB activity and IκBα promoter activity. Tet-on Wip1 inducible H1299 cells transfected with either A) the NF-κB or B) IκBα reporter construct were incubated with doxycycline for the indicated time to induce Wip1 expression and luciferase activity was measured. C) NF-κB activity or D) IκBα promoter activity was measured in H1299 cells either without Wip1 expression (“-dox”) or with Wip1 expression by incubation of doxycycline for twenty-four hours (“+dox”). NF-κB and IκBα promoter activity were normalized to EF1α promoter activity.
in IκBα promoter activity with an increase in Wip1 expression (similar to the NF-κB-driven luciferase activity) supports the idea that IκBα promoter activity is a good measure of NF-κB activity and that Wip1 expression enhances NF-κB activity. The reason for such a dramatic increase in NF-κB activity at five hours post-doxycycline addition and not at other times is unknown, but may be due to a peak in Wip1 expression. If this were the case, then measurement of NF-κB activity between the time of doxycycline addition and five hours post-addition would reveal a steady incline in NF-κB activity (in parallel with increasing Wip1 expression), and this should be tested in the future.

Analysis of NF-κB-driven luciferase activity and IκBα promoter activity at twenty-four hours after doxycycline addition (when Wip1 expression is stable) showed different results. While there is no significant difference in basal NF-κB-driven luciferase activity between Wip1 expressing (“+dox,” Figure 34C) and non-Wip1 expressing (“-dox,” Figure 34C) cells, there is a significant increase in basal IκBα-driven luciferase activity in Wip1 expressing cells (“+dox,” Figure 34D) compared to non-Wip1 expressing cells (“-dox,” Figure 34D). The reason for this is unclear, but may be due to an increased sensitivity of the IκBα reporter construct compared to the NF-κB-driven reporter construct. Nevertheless, these data taken together indicate that measurement of IκBα promoter activity using the construct described here is an effective measure of NF-κB activity and may not be the best way to measure the effect Wip1 has on regulating IκBα transcription.
**Figure 35**

**Wip1 expression increased TNFα-induced NF-κB activity.** A) The NF-κB or B) IκBα reporter construct was co-transfected with the EF1α reporter construct in tet-on Wip1 inducible H1299 cells expressing Wip1 or not. Basal (“0”) or TNFα-induced (three hours after stimulation, “3”) luciferase activity was measured. C) IκBα protein was measured by immunoblot analysis in H1299 cells with or without Wip1 expression. Protein was harvested without stimulation or at the indicated time after TNFα addition. (“+dox” indicates Wip1 expressing cells, and “-dox” indicates non-Wip1 expressing cells)
6.2.9 Wip1 reduces basal IκBα expression and enhances TNFα-induced NF-κB activity.

Basal and TNFα-induced NF-κB activity, IκBα promoter activity, and IκBα protein levels were next measured in the tet-on Wip1 inducible H1299 cells since these cells can be induced to express more moderate Wip1 expression levels than observed in cells that have ectopic Wip1 over-expression. Wip1 expression increases NF-κB activity three hours after TNFα stimulation as seen by an increase in the NF-κB- (“+dox,” Figure 35A) and IκBα-driven (“+dox,” Figure 35B) luciferase activity compared to the non-Wip1 expressing control cells (“-dox,” Figure 35A and B), demonstrating that Wip1 expression augments TNFα-induced NF-κB activity in H1299 cells.

Immunoblot analysis of IκBα protein was also performed in the H1299 cells with and without TNFα stimulation. As seen in Figure 35C, basal IκBα protein levels are decreased in Wip1-expressing cells (“+dox,” Figure 35C) compared to non-Wip1 expressing cells (“-dox,” Figure 35C).
Figure 35C). Upon TNFα stimulation, IκBα protein is degraded in order to activate NF-κB, which is seen by a decrease in IκBα protein levels thirty minutes after stimulation regardless of Wip1 expression (Figure 35C). Additionally, IκBα protein levels are higher at two hours post-TNFα stimulation in Wip1 expressing cells (“+Dox,” Figure 35C) compared to the control cells (“-dox,” Figure 35C). This is consistent with the idea that Wip1 enhances NF-κB activity after TNFα stimulation since there are higher levels of IκBα protein two hours after stimulation in cells expressing Wip1.

6.3 Discussion

The results described in this chapter show that Wip1 can enhance basal, IR-induced, and TNFα-induced NF-κB activity. Furthermore, Wip1 increases NF-κB activity through a similar mechanism as other known stimuli, i.e. through inhibition of IκBα. However, Wip1 appears to have a different effect on NF-κB-induced IκBα expression in the systems used in these studies, which include ectopic overexpression of Wip1 by transient transfection and more moderate levels of Wip1 expression in the tet-on Wip1 inducible H1299 cell line (Figure 36). As summarized in Figure 37A, controlled expression of Wip1 in the tet-on Wip1 inducible H1299 cells inhibits IκBα expression and activates NF-κB, which enhances the expression of IκBα. IκBα accumulates in the cell and inhibits NF-κB in a negative feedback loop, which reduces NF-κB activity back to basal levels. Also, Wip1 enhances NF-κB activation after stimulation, for example by IR exposure or TNFα stimulation, and IκBα accumulates. On the other hand, ectopic over-expression of Wip1 by transient transfection results in higher expression levels than
seen in the tet-on Wip1 inducible H1299 cells with doxycycline supplemented in the media (Figure 36, Figure 37B). Wip1 over-expression inhibits IκBα expression similar

**Figure 37**

**Proposed model of Wip1 activation of NF-κB.** A) Wip1 expression at moderate levels such as those seen in doxycycline treated Wip1 inducible H1299 cells inhibit IκBα expression and activate NF-κB. Active NF-κB enhances IκBα expression, which returns NF-κB activity to basal levels. B) High Wip1 over-expression such as that seen in transient transfection of a Wip1 expression vector also inhibits IκBα expression and activates NF-κB. In this system, NF-κB-induced IκBα expression is also inhibited, which eliminates the negative feedback loop and results in an increase basal NF-κB activity and a decrease in basal IκBα expression.

to that seen in H1299 cells, which results in NF-κB activation and initiation of the NF-κB/ IκBα negative feedback loop. However, the high Wip1 expression levels may be
sufficient to inhibit IκBα expression induced by active NF-κB (basally and after exogenous stimulation, Figure 37B). In other words, Wip1 over-expression by transient transfection of an expression vector (in combination with stress-induced endogenous Wip1) could be at high enough levels to eliminate the NF-κB/ IκBα negative feedback loop. This difference may explain the reason for the high basal NF-κB activity twenty-four hours after transient transfection but not after doxycycline treatment in the H1299 cells. This may also explain the low levels of IκBα expression in Wip1 over-expressing PC-3 cells after IR exposure (Figure 31).

Another possible reason for the difference in NF-κB activation by Wip1 in these cells is that it may be tissue-specific, and regulation of NF-κB activity is dependent on tissue type (67,76,93). Both PC-3 and DU-145 cells are of prostate origin, whereas H1299 cells are lung epithelial cells. Moreover, NF-κB is constitutively activated in PC-3 and DU-145 cells by various mechanisms (135,140-143), whereas NF-κB activity has been mainly studied in response to stimuli such as TNFα in H1299 cells (144-146). The differences in basal NF-κB activation or the tissue origin may account for the difference in Wip1-induced NF-κB activation in these cells.

It is interesting that the kinetics of TNFα-induced NF-κB activation in the PC-3 and DU-145 cell lines is different (Figure 29). NF-κB activation can be seen dramatically starting at one hour after stimulation in DU-145 cells, whereas NF-κB activation is less dramatic and begins to be seen at two hours in PC3 cells. Despite these differences, Wip1 over-expression only augments (and does not change) the pattern of
TNFα-induced NF-κB activation in these cell lines. This fact supports the notion that Wip1 affects a shared aspect of NF-κB activation, which these studies indicate is at the level of IκBα expression.

These studies indicate that Wip1 decreases IκBα mRNA stability. However, whether Wip1 affects IκBα transcription is inconclusive. The results from the IκBα promoter activity studies are difficult to interpret in this respect because of the negative feedback loop between NF-κB and IκBα. For example, Wip1 may be inhibiting IκBα transcription by regulating cofactors or other proteins that require sequences in the IκBα promoter region not included in the reporter construct used in this study. It is clear, however, that the IκBα reporter vector used here is a good tool to indirectly measure NF-κB, and is not a good way to measure Wip1 regulation of IκBα promoter activity. Therefore, Wip1 regulation of IκBα promoter activity must be tested using a different approach in the future.

The fact that there is only a significant increase in IκBα promoter activity but not in NF-κB-driven luciferase activity by Wip1 in H1299 cells is conflicting (Figure 34). The reasons for this discrepancy are unclear. However, since Wip1 expression is at a lower level compared to ectopic Wip1 over-expression, more sensitive assays may be necessary to detect differences in NF-κB activity between Wip1 expressing and non-Wip1 expressing H1299 cells. In this light, using the IκBα reporter construct may be a more sensitive way of measuring NF-κB activity than using the NF-κB-driven reporter construct. The NF-κB-driven reporter construct contains about 4-5 repeats of the κB site
(GGGAATTTCC) upstream of the luciferase gene, whereas the IκBα reporter construct contains part of the promoter sequence upstream of the NFκBIA gene. The additional sequence in the IκBα reporter construct may result in a heightened response to NF-κB activation by allowing coactivators to bind the regions near the κB site, which may enable smaller changes in NF-κB activity to be seen when using this vector.

The conclusion of these studies (Wip1 enhancement of NF-κB activity) is in direct contrast to a report by Chew et al, which describes Wip1 as a negative regulator of NF-κB signaling by dephosphorylation of S536 on the p65 NF-κB subunit. It is not surprising that Wip1 in some contexts may inhibit NF-κB, while in other contexts enhance NF-κB signaling. NF-κB activity regulation, including p65 phosphorylation, is extremely complex and has been shown to be dependent on the type of stimulus and tissue (67,76,93). An example of this is that the p65 subunit of NF-κB differentially regulates Wip1 expression depending on stimulus as shown in Chapter 4. Our results presented here, therefore, illustrate the complexity of NF-κB regulation by Wip1.

Wip1 enhancement of NF-κB activity also fits well with known aspects of Wip1 signaling. For example, after stress, such as IR exposure, Wip1 inhibits pro-apoptotic signaling by stabilizing MDM2 and inactivating p53. Adding activation of NF-κB as a function of Wip1 after stress complements the inhibition of pro-apoptotic signaling, since NF-κB promotes survival after stress (79,98,100). In other words, Wip1 promotes cell survival after stress by inhibiting apoptosis by inactivating p53 (directly and indirectly
through MDM2 stabilization) and enhancing pro-survival signaling by activating NF-κB, which facilitates the return of the cell to homeostasis.
Chapter 7. Discussion

7.1 Signaling between NF-κB and Wip1 depends on context

NF-κB signaling is complex, and NF-κB activation can occur by different mechanisms depending on tissue type and the type of stimulus. For example, certain phosphorylation sites of p65 are important after some stimuli and not others (147). Additionally, the same protein can exert different effects on NF-κB depending on the stimulus. For example, IR exposure induces activation of NF-κB by ATM phosphorylation of NEMO (the regulatory subunit of the upstream NF-κB activator) (69,148), but replication stress represses NF-κB activation by ATM binding to, but not phosphorylating, NEMO (149). Not only does NF-κB signaling depend on the type of stimulus, but it also depends on the tissue type. For example, over-expression of p65 results in apoptosis in transformed pro-B-cells but not in immature B-cells (150).

Given the complexity and context dependence of NF-κB signaling, it is not surprising that Wip1 can both negatively and positively regulate NF-κB. Studies performed by Chew et al describe a physiological function for Wip1 – to inhibit NF-κB and turn-off inflammation. However, Wip1 enhancement of NF-κB after genotoxic stress fits with the known Wip1 function of reversing stress signaling once damage is repaired. In other words, Wip1 can ensure the survival of a cell after stress not only by inhibiting apoptosis and cell cycle arrest but also by augmenting survival cues through NF-κB activation (Figure 38).
Wip1 signaling after genotoxic stress. *Contributions of the studies from this project add to Wip1 signaling. Wip1 is upregulated by NF-κB; Wip1 is downregulated by NF-κB; Wip1 inhibits NHEJ through DNA-PKcs; Wip1 promotes anti-apoptotic NF-κB signaling.

Differential NF-κB activation in different cellular settings has a major affect on the genes targeted by NF-κB. Therefore, there appear to be different sets of NF-κB target genes for each stimulus. Likewise, genes can be regulated differently by NF-κB depending on the cell type and stimulus (67,76,93). For example, even though NF-κB signaling in most cases is anti-apoptotic by upregulating the transcription of anti-apoptotic genes, NF-κB has been shown to be pro-apoptotic by repressing these same anti-apoptotic genes after certain stimuli. This is the case with the anti-apoptotic protein,
X-IAP. X-IAP is induced by NF-κB after TNFα stimulation, but it is repressed by NF-κB after UVR exposure [Campbell, 2004 #995]. Wip1 appears to be another example of a NF-κB target gene that is differentially regulated depending on the stimulus. As explained in Chapters 3 and 4, NF-κB upregulates Wip1 expression after TNFα but also decreases Wip1 expression after IL-1β. Even within the same system, Wip1 is differentially regulated – Wip1 expression is inhibited at early times after LPS stimulation in splenic lymphocytes and is then upregulated at later times, and NF-κB is presumably the main regulator in both cases. Taken together the data presented here shows that Wip1 regulation of NF-κB and NF-κB regulation of Wip1 is complex and context dependent, which suggest that the functional consequences of this signaling is also context dependent.

7.2 NF-κB, DNA-PK, and Wip1 signaling – implications in cancer

The signaling described in this study - NF-κB positive regulation of Wip1 expression, Wip1 inhibition of DNA-PKcs and NHEJ, and Wip1 augmentation of stress-activated NF-κB – has significant implications in cancer cells. NF-κB upregulation of Wip1 transcription may be a means by which Wip1 is over-expressed in cancer cells (Figure 39). Wip1 is a target of p53; however, p53 is often either non-functional or found at very low levels in tumor cells since it is a tumor suppressor (151). Additionally, breast tumors that have Wip1 over-expressed also have very low levels of p53 expression (8). Therefore, high Wip1 expression must be maintained in cancer cells by other means besides through p53. ERα has been shown to play this role in breast
The impact of Wip1 signaling on cancer. *Wip1 promotes tumorigenesis by three main mechanisms: 1) augmentation of NF-κB, 2) inhibition and enhancement of pro-apoptotic and pro-survival stress signaling, respectively, and 3) inhibition of tumor suppressors. Also, NF-κB induces Wip1 expression in cancer cells. Contributions of the studies from this project are highlighted in red.

cancer cells (30); however, Wip1 is over-expressed in hormone-independent cancers (6). On the other hand, NF-κB has been shown to be active in a variety of cancers (92), and NF-κB could be a major mechanism by which Wip1 expression is induced in cancer cells (Figure 39 and Figure 39). We show that this is the case in MCF-7 breast carcinoma cells in Chapter 3, since NF-κB was found to be constitutively bound to the PPM1D
promoter region and responsible for a large amount of basal Wip1 expression in these cells. Therefore, inhibition of NF-κB could also inhibit Wip1 expression in cancer cells, which may be an important factor to consider regarding the use of NF-κB inhibitors as chemotherapeutic agents.

Inhibition of NHEJ and activation of NF-κB may be other mechanisms by which Wip1 promotes tumorigenesis by enhancing genomic instability (Figure 39). In a normal functioning cell, DNA-PK along with other proteins ensures that DNA damage is repaired by NHEJ before the cell enters the cell cycle. If DNA-PK is inhibited prematurely by Wip1 before DNA damage is repaired after genotoxic stress, gene mutations may occur. Additionally, NF-κB activation after genotoxic stress results in the upregulation of anti-apoptotic genes and enhances survival. After genotoxic stress, cells that over-express Wip1 may have premature termination of the DNA damage response, including the inhibition of NHEJ, along with increased survival cues through activation of NF-κB. In this case, cells would be able to enter the cell cycle and replicate damaged DNA, which may genomic instability and tumorigenesis (Figure 39).

In addition to increasing genomic instability, Wip1 activation of NF-κB may help cancer progression (Figure 39). Activation of NF-κB leads to the activation or repression of several target genes, which results in enhanced survival and proliferation in most cases (82). This has been shown to be the case in many cancer cells when NF-κB is activated constitutively and after chemotherapy and radiotherapy (78,91,135,143,152,153). Since Wip1 is over-expressed in many cancers, augmentation of NF-κB activity by Wip1 may
be another mechanism by which Wip1 promotes tumor cell survival and proliferation. Additionally, Wip1 may increase NF-κB activation after chemotherapy and radiotherapy, which may enhance the radioresistance of tumor cells, especially those in which Wip1 is over-expressed.

7.3 Wip1 helps return cells to homeostasis after stress: new roles for NF-κB and DNA-PKcs

As described in the Chapter 1, Wip1 inhibits stress-induced apoptosis, cell cycle arrest and DNA repair by inactivating stress signaling proteins such as p53, p38, and ATM. Given this role of Wip1 in stress signaling, the physiological function of Wip1 is to facilitate the return of cells to homeostasis after stress (6). The studies from this project add to the mechanisms by which Wip1 performs this role.

Although p53 is a major regulator of Wip1 transcription after genotoxic stress (5), there are likely other factors responsible for Wip1 expression regulation since Wip1 has been shown to be upregulated independent of p53 in some circumstances (see Introduction). The discovery that Wip1 inhibits inflammation by dephosphorylating and inactivating the p65 NF-κB subunit (46) highlighted the fact that Wip1 may also be regulated by NF-κB and that Wip1 and NF-κB could be acting in a negative feedback loop. This seemed to make sense, since Wip1 and p53 act in a negative feedback loop, i.e. p53 induces Wip1 expression and Wip1 inactivates p53, and both p53 and NF-κB are stress-activated transcription factors. The results presented here show that NF-κB is a regulator of Wip1 transcription after different types of stimuli, including genotoxic stress;
although, as described above, the interaction between NF-κB and Wip1 is not limited to a simple negative feedback loop.

Wip1 regulation of NF-κB adds to the complex network of feedback loops, which depend on the stimulus and cell type. The results presented here show that NF-κB enhances Wip1 expression in response to inflammatory signaling such as TNFα, and Chew et al show that Wip1 inhibits TNFα–induced NF-κB activity to turn-off inflammatory signaling. However, the results from Chapter 6 show that in some cell types, Wip1 enhances TNFα–induced NF-κB activity, which may be relevant in a cancer setting since low levels of TNFα have been shown to promote tumorigenesis. Additionally, NF-κB positively regulates Wip1 after genotoxic stress, and Wip1 enhances genotoxic stress-induced NF-κB activity, which may promote survival of cells once damage is repaired (Figure 38). Therefore, in different circumstances, some of these feedback loops between Wip1 and NF-κB are additional mechanisms by which Wip1 returns the cell to homeostasis after stress, whether the stress is an immunological challenge or genotoxic stress.

The reversal of stress signaling by Wip1 is well-characterized after genotoxic stress and was previously known to be involved in the inhibition of apoptosis and reversal of cell cycle arrest. The results presented here, however, add new aspects to Wip1 signaling after genotoxic stress that help to return the cell to homeostasis (Figure 38). Not only does activation of NF-κB enhance the survival cues to ensure an escape from
apoptosis, but inhibition of DNA-PK by Wip1 provides a mechanism by which Wip1 turns off NHEJ, one of the most important DNA repair processes (Figure 38).

In conclusion, the main goal of this project was to identify novel regulation of Wip1 expression and Wip1 signaling after stress, since these findings would contribute to the understanding of the functions of Wip1 in both a physiological and tumorigenic setting. As a result, new roles for NF-κB and DNA-PK in Wip1 signaling were identified, which have direct implications in the understanding of Wip1 oncogenic functions and the treatment of human cancer.
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